

Selection of probiotic lactic acid bacteria for horses based on *in vitro* and *in vivo* studies

By

Marlie Botha

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Supervisor: Prof. L.M.T. Dicks
Co-supervisor: Dr. M. Botes
Faculty of Science
Department of Microbiology

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DECLARATION

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SUMMARY

The equine gastro-intestinal tract (GIT) is a relatively unexplored niche concerning the presence of natural microbiota. Studies have shown that disruption of the microbial population naturally present in the GIT leads to the onset of several forms of gastro-intestinal disorders. To maintain a balanced microbiota, probiotic bacteria need to be administered at specific levels. Beneficial microorganisms assist with digestion of the feed, absorption of nutrients from the GIT, strengthens the immune system and improves the animal's growth. Various combinations of lactic acid bacteria (LAB) have been administered to horses, but have failed to benefit the host in any of the latter criteria. The screening for alternative strains with probiotic properties is thus necessary.

Two strains (*Lactobacillus equigenerosi* Le1 and *Lactobacillus reuteri* Lr1) were originally isolated from horse faeces. *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA, both bacteriocin-producing strains, were isolated from sorghum beer and soy beans, respectively. All four strains survived growth at acidic conditions (pH 3) and the presence of 0.5%, 1.0% and 1.5% (w/v) bile salts. *L. reuteri* Lr1 was the most resistant to these conditions. All strains adhered to buccal (cheek) epithelium cells sampled from horses. *L. equigenerosi* Le1 and *E. mundtii* ST4SA, however, invaded the cells, but without visible signs of disrupting the cells. None of the strains contained genes encoding adhesion to collagen (Ace), resistance to vancomycin A, B and C, or, production of aggregation substance (AS), cytolysin (Cyl) and, non-cytolysin (β hemolysin III), suggesting that they are non-virulent. Of all strains, *L. equigenerosi* Le1 competed the best with *Clostridium* sp. C6 for adherence to epithelial cells. *L. equigenerosi* Le1 and *L. reuteri* Lr1, showed the highest level of co-aggregation with *Clostridium* sp. C6.

When the four strains were administered to horses over a period of 10 days, *L. reuteri* Lr1 was retained the longest (8 days) in the GIT. The numbers of viable cells of *Clostridium* spp. and *Salmonella* spp. remained constant during administration of the four strains. Blood analyses showed no negative effects from administering the strains. Total white blood cell counts remained unchanged. However, a small but tentative increase in neutrophil and eosinophil cell numbers has been recorded, suggesting that the LAB may have elicited a mild, transient, intolerance reaction. The glucose, lactate and urea levels decreased during administration with the four LAB strains.

OPSOMMING

Die spysverteringstelsel (SVS) van die perd is 'n relatief onbekende nis wat die voorkoms van natuurlike mikrobiota betref. Studies het getoon dat versteuring van die natuurlike mikrobiese populasie in die SVS aanleiding kan gee tot die ontwikkeling van menige vorms van gastro-intestinale ongesteldhede. Om 'n gebalanseerde mikrobiota te verseker, moet probiotiese bakterieë teen 'n spesifieke vlak toegedien word. Voordelige mikroorganismes bevorder vertering en absorpsie van nutriënte vanaf die SVS, versterk die immuunsisteem en bevorder die groei van die dier. Verskeie kombinasies van melksuurbakterieë is reeds aan perde toegedien, maar sonder ooglopende voordele vir die dier. Die soeke na alternatiewe stamme met probiotiese eienskappe is dus noodsaaklik.

Twee melksuurbakterieë (*Lactobacillus equigenerosi* Le1 en *Lactobacillus reuteri* Lr1) is oorspronklik uit perdemis geïsoleer. *Lactobacillus plantarum* 423 en *Enterococcus mundtii* ST4SA, beide bakteriosienproduserende stamme, is afsonderlik van sorghumbier en sojabone geïsoleer. Al vier spesies groei by lae pH (pH 3) en in die teenwoordigheid van 0.5%, 1.0% en 1.5% (m/v) galsoute. *L. reuteri* Lr1 is die mees bestand onder hierdie toestande. Al vier stamme het aan wang epiteelselle van perde geheg. *L. equigenerosi* Le1 en *E. mundtii* ST4SA het egter die epiteelselle binnegedring, maar sonder opsigtelike vernietiging van die selle. Nie een van die stamme besit gene wat kodeer vir aanhegting aan kollageen (Ace), bestandheid teen vankomisien A, B en C, of produksie van, sel-aggregasie (AS), sitolisien (Cyl) en nie-sitolisien (β -hemolisien III), wat daarop dui dat hulle nie-virulent is. Van al die stamme het *L. equigenerosi* Le1 die beste met *Clostridium* sp. C6 vir aanhegting aan epiteelselle gekompeteer. *L. equigenerosi* Le1 en *L. reuteri* Lr1, het die beste vlak van ko-aggregasie met *Clostridium* sp. C6 getoon.

Met die toediening van 'n kombinasie van die vier stamme aan die perde oor 'n periode van 10 dae, het *L. reuteri* Lr1 die langste retensie (8 dae) in die SVS getoon. Die aantal lewende selle van *Clostridium* spp. en *Salmonella* spp. het konstant gebly tydens toediening van die vier stamme. Toediening van die vier stamme het geen negatiewe effek getoon met resultate verkry van bloed analyses nie. Die totale witbloed seltellings het onveranderd gebly. 'n Klein, maar tentatiewe, toename in neutrofiel- en eosinofiel selgetalle is waargeneem, wat daarop dui dat die melksuurbakterieë 'n geringe allergiese reaksie teweeggebring het. Die glukose, laktaat en ureum vlakke het gedaal tydens die toediening van die vier melksuurbakterie stamme.

PREFACE

The introduction to the thesis, Chapter 1, provides a short background, motivation and outlines the basic objectives of this study. The chapter has been written according to the style of *Applied and Environmental Microbiology*.

The literature review, “The Equine Gastro-intestinal Tract: An Overview of the Microbiota, Disease and Treatment”, is an overview of the natural microbiota within the equine gastro-intestinal tract (GIT). Gastro-intestinal diseases caused by a disrupted microbial population and treatment, including the use of probiotics, are discussed. This chapter has been prepared for submission to *Veterinary Microbiology*.

Chapter 3, “Probiotic Potential of *Lactobacillus equigenerosi*, *Lactobacillus reuteri*, *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA in Horses” and Chapter 4, “Survival of Lactic Acid Bacteria through the Intestine and Physiological Changes Recorded when Administered to Horses” have been written according to the style of *Applied and Environmental Microbiology*.

The general discussion and conclusions of this study is presented in Chapter 5. This chapter has been written according to the style of *Applied and Environmental Microbiology*.

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CHAPTER 1

INTRODUCTION

Written according to *Applied and Environmental Microbiology*

INTRODUCTION

The microbiota of the horse and microbial interactions in the gastro-intestinal tract (GIT) is a relatively unexplored research field. GIT disorders and diseases such as lactic acidosis and colic have been linked to an imbalanced gut microbial population and is most likely the cause of a carbohydrate overload (7, 14, 21, 25). Antibiotics are widely used to treat diseases in animals (29). A rise in antibiotic-resistant bacteria (27) and reported disruption of the microbial populations when administering antibiotics (2, 3, 16, 17, 18) has resulted in the use of probiotics as suitable alternatives.

Probiotics are live microbial feed supplements that are beneficial to the host when administered at specific cell numbers (11). In farm animals, the administration of probiotics increased growth rate (23, 28), the digestion and absorption of nutrients within the intestine (1), and prevents the onset of disease (12, 19). Most probiotic supplements contain lactic acid bacteria (LAB) (13).

Some of the most important criteria for a microorganism to be classified as a probiotic are the ability to survive passage through the GIT, more specifically the acidic stomach conditions (23). The strains also have to survive fluctuations in bile salts in the GIT (10). Furthermore, to colonize the GIT, strains need to adhere to the epithelium or mucus in order to prevent pathogens from adhering (13, 15, 26). Another form of competitive exclusion is removal of the pathogen from the GIT by binding and co-aggregation (8).

Selected strains first have to be evaluated *in vitro* for their potential to be administered as probiotics. Various GIT models have been developed to perform these tests (5, 20, 22, 30). *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA survived conditions simulating the GIT (5) and studies with Caco-2 cell lines have shown that the strains have colonising properties (4). *In vivo* studies are normally done with mice and rats as models (6, 9, 24, 30).

In this study, four strains have been evaluated for probiotic properties. *Lactobacillus equigenerosi* Le1 and *Lactobacillus reuteri* Lr1 have been isolated from horse faeces. *L. plantarum* 423 and *E. mundtii* ST4SA, both bacteriocin-producing strains, have been isolated from sorghum beer and soybeans, respectively. The objectives of the current study and questions raised were as follows:

In vitro studies:

- Do the four lactic acid bacteria survive low pH and have the ability to grow in the presence of bile salts?
- Do the strains contain virulence genes, in other words are they safe to use as probiotics?

- Do the strains have the ability to adhere to viable epithelium cells?
- What effect do the strains have on non-viable epithelium cells?
- What effect proteolytic enzymes have on strain adherence?
- Do the strains compete with pathogens for adhesion to the GIT?
- Do the strains auto-aggregate or co-aggregate with pathogens?

In vivo studies:

- Do the four strains survive conditions in the equine GIT?
- Do the strains cause a shift in the microbial population of the microbiota in the GIT when administered on a daily basis?
- Do the strains have any negative effect on horses?

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CHAPTER 2

The Equine Gastro-intestinal Tract: An Overview of the Microbiota, Diseases and Treatment

Prepared for submission to *Veterinary Microbiology*

The Equine Gastro-intestinal Tract: An Overview of the Microbiota, Diseases and Treatment

M. Botha, M. Botes and L.M.T. Dicks

Department of Microbiology, University of Stellenbosch, Stellenbosch 7600, South Africa

Abstract

The horse is a hindgut fermenter, *i.e.* most microbial activities takes place in the large intestine which constitutes approximately 60 percent of the gastro-intestinal tract. The feed reaches the large intestine after approximately 3 h and is fermented for 36-48 h in the caecum. This rate of transition is only possible if the roughage component of the feed is kept optimal. A diet rich in starch leads to an imbalance in intestinal microbiota, which may lead to colic and often death. Lactic acid bacteria form a major constituent of the microbiota in the gastro-intestinal tract, especially in the large intestine, and produce most of the volatile fatty acids needed for energy. Production of antimicrobial compounds, including antimicrobial peptides (bacteriocins) may prevent the growth of pathogens and keep a healthy microbial balance in the gastro-intestinal tract. Lactic acid bacteria may also play a role in stimulation of the immune system. This review focuses on the microorganisms in the equine gastro-intestinal tract and their role in health and disease.

Key words: Equine GIT, microbiota, diseases, treatment, lactic acid bacteria, probiotics

Introduction

The horse (*Equus caballus*) is a monogastric, hindgut fermenting animal, *i.e.* most of the feed is degraded in the caecum and colon (Fig. 1). Production of large quantities of saliva (10- 12 L per day) helps to transport the feed through a 1.2-1.5 m long oesophagus (Cunha, 1991) and buffers the digesta (Frape, 2010). The oesophagus enters the stomach at the oesophageal section (Fig. 2). This part of the stomach is non-glandular (Pilliner, 1993), but pepsin and other proteolytic enzymes are secreted by glands in the pyloric section (Frape, 2010; Pilliner, 1993). Transition of digesta through the stomach is relatively rapid, although a large portion remains for 2-6 h in the anaerobic fundic (lower) section of the stomach. Carbohydrates are fermented to lactic acid and the pH of the digesta decreases to approximately 2.6 (Frape, 2010). Most of the enzymatic breakdown and absorption of digesta takes place in the small intestine (Frape, 2010; Pilliner, 1993). As soon as the acidic digesta reaches the duodenum, the pH is neutralized to 7.0 or 7.4 (Frape, 2010; Kern et al., 1974) by bile secreted from the liver (the horse does not have a gall bladder) and fats are emulsified (Colville and Bassert, 2008; Cunha, 1991; Frape, 2010). Proteins and fat are digested to produce fatty acids and glycerol (Frape, 2010). Soluble carbohydrates are hydrolyzed by α -amylase and α -glucosidase (Frape, 2010) to lactic acid which are absorbed, together with fatty acids, vitamins and minerals (Frape, 2010; Pagan, 1998). Digesta reaches the caecum and colon approximately 3 h after feeding (Frape, 2010). The caecum of 25-35 L (Frape, 2010; Pilliner, 1993) has two valves situated relatively close to each other (Frape, 2010). The ileum enters at the position of the first valve (Frape, 2010). Further passage to the colon is through the second valve (Frape, 2010). The motility and capacity of the caecum increase during feeding to optimise interaction between the bacteria and digesta (Frape, 2010). The pH of the caecum and colon is approximately 6.0 and forms the ideal condition for anaerobic bacteria, fungi and protozoa to degrade hemicelluloses and pectins (Bonhomme-Florentin, 1988; Kern et al., 1974). Complex carbohydrates such as cellulose are fermented (Frape, 2010; Pagan, 1998; Pilliner, 1993), and vitamins B and K and essential amino acids are synthesized (Frape, 2010; Pagan, 1998). Residual carbohydrates are starch that may end

up in the large intestine is slowly fermented, and when present in excess quantities, may favour the growth of amylolytic bacteria. This causes an imbalance in the microbial population that may lead to lactic acidosis (production of an excess amount of lactic acid) or colic (Clarke et al., 1990; Garner et al., 1977; Milinovich et al. 2005; Rowe et al., 1994). If the dry matter content of the feed is too high (low carbohydrate levels), non-lactic acid bacteria dominate, the pH increases, and CO₂ and volatile fatty acids are produced, all of which lead to severe gastric problems (Frape, 2010). It is thus important to ensure that the microbiota in the gastro-intestinal tract (GIT) is always in a well-balanced state.

This review gives an overview of the beneficial microorganisms and pathogens in the GIT of horses and emphasises the role that lactic acid bacteria play in maintaining a healthy intestine. The probiotic properties of lactic acid bacteria are discussed and the ability of intestinal bacteria acquiring resistance to antibiotics is investigated.

Microbiota in the gastro-intestinal tract

Compared to other animals and humans, little research has been conducted on the microbiota in the gastro-intestinal tract of horses. *Streptococcus equi* isolated from the cheek and tongue epithelium cells of ponies has been associated with strangles, a mouth and nose disease (Srivastava and Barnum, 1983). The oesophagus is colonized by obligately and facultatively anaerobic bacteria (Meyer et al., 2010). Predominant species include *Actinobacillus equuli*, *Bacteroides* spp., *Fusobacterium* spp., *Prevotella* spp. and streptococci (Bailey and Love, 1991; Meyer et al., 2010). Yeasts are present, but in low numbers (Meyer et al., 2010).

Various studies on intestinal microbiota focussed on the microbial population in the stomach. *Lactobacillus agilis*, *Lactobacillus crispatus*, *Lactobacillus reuteri* and *Lactobacillus salivarius* have been isolated from the oesophageal section (Yuki et al., 2000) and *Lactobacillus delbrueckii*, and *L. salivarius* from the more anaerobic fundic section (Al Jassim et al., 2005). Some strains are,

however, host specific as shown with adhesion studies conducted on epithelial cells (Yuki et al., 2000). *Lactobacillus* and *Streptococcus* spp. isolated from the fundic section of the stomach (10^8 - 10^9 CFU/ml) represents almost the entire population of anaerobic bacteria (de Fombelle et al., 2003; Frape, 2010). Lactobacilli are the most prevalent (de Fombelle et al., 2003).

The mucosae and lumen of the duodenum, jejunum and ileum contains between 10^6 and 10^7 viable bacteria per mL, of which most have proteolytic activity (Mackie and Wilkins, 1988). High cell numbers (10^6 - 10^9 cfu/ mL) of anaerobic bacteria, especially streptococci, have also been isolated (de Fombelle et al., 2003). *Candida*, *Clostridium*, *Proteus*, *Pseudomonas* and *Staphylococcus* spp. are also present, but in low numbers (Julliand, 2005). Glands in the large intestine secrete mucus, but no digestive enzymes (Frape, 2010). The caecum contains mainly amylolytic, cellulolytic, glucolytic, hemicellulolytic, lactate fermenting and proteolytic bacteria (Mackie and Wilkins, 1988). Of these, proteolytic bacteria such as *Streptococcus bovis*, *Streptococcus equinus* and *Bacteroides* spp. are the most dominant (Julliand, 2005; Kern et al., 1973). It is, however, important to note that only 20% of the total number of bacteria in the large intestine is proteolytic and that most of the protein digestion takes place in the small intestine (Frape, 2010). Only two isolates had urease activity and were identified as staphylococci (Maczulak et al., 1985).

A large variety of microorganisms, all with some role in digestion, have been isolated from the caecum and colon. The caecum contains approximately 10^9 bacteria per gram ingesta (Mackie and Wilkins, 1988). Lactate-utilizing bacteria within the caecum and colon range between 10^5 and 10^6 cfu/ mL (de Fombelle et al., 2003). These lactate-utilizing bacteria were identified by Julliand (Julliand, 2005) as being predominantly *Megasphaera* sp. and *Veillonella* sp. The most important cellulolytic and fibrolytic bacteria according to Daly *et al.* (Daly et al., 2001) are the *Butyrivibrio* spp., the *Clostridium* spp., the *Eubacterium* spp. and *Ruminococcus* spp. Cellulolytic bacteria inhabit the caecum more often than the colon (Frape, 2010). *Ruminococcus flavefaciens* is the predominant cellulolytic bacterium within the caecum, followed by *Fibrobacter succinogenes* and

Ruminococcus albus (Julliand et al., 1999). Anaerobic fungi have also been isolated from the caecum (Orpin, 1981) and Julliand (Julliand, 2005) reported 10^1 and 10^4 zoospores per mL content. *Trichosporon cutaneum* has been isolated from the caecum (Uden et al., 1958). The pathogen, *Cryptococcus neoformans*, was isolated from a healthy horse and it is thought that the horse plays a role in distribution of this yeast (Uden et al., 1958).

The colon contains between 10^5 and 10^8 viable bacteria per mL (de Fombelle et al., 2003; Mackie and Wilkins, 1988). *Butyrivibrio fibrisolvens*, *Campylobacter lanienae*, *Clostridium barati*, *Ruminococcus flavefaciens* and *Streptococcus bovis* have been isolated from the large intestine (Daly et al., 2001). Lactic acid bacteria isolated from faeces included *Lactobacillus delbrueckii*, *L. salivarius* (Al Jassim et al., 2005; Morita et al., 2009), *Lactobacillus mucosae* (Al Jassim et al., 2005), *Lactobacillus equi* (Morotomi et al., 2002, Morita et al., 2009), *Lactobacillus equigenerosi*, *Lactobacillus hayakitensis*, *Lactobacillus buchneri*, *Lactobacillus vitulinus* (Morita et al., 2009), *Lactobacillus crispatus*, *Lactobacillus johnsonii* and *L. reuteri* (Morotomi et al., 2002). Enterococci have been isolated from the rectum, including Vancomycin-A resistant strains of *Enterococcus casseliflavus* and *Enterococcus faecium* (de Niederhäusern et al., 2007). Virulent strains of *Rhodococcus equi* (Bourgeois-Nicolaos et al., 2006) and *Helicobacter equorum* (Moyaert et al., 2007) have also been isolated from faeces. Heitmann et al. reported the presence of *Mycoplasma* spp. in faeces (Heitmann et al., 1982).

The Archaea present in horses constitute approximately 3.5% of the total microbial cell numbers (Yamano et al., 2008). The methanogen population in the caecum is approximately 10^5 /ml (Vermorel et al., 1997). Fifty-three thermophilic methanogens were isolated from faeces (Kitaura et al., 1992). The Archaea fall within the *Methanobrevibacter* group (Lange et al., 2005; Willing, 2011), sharing 16S rRNA gene similarity with *Methanobrevibacter smithii* (Willing, 2011).

Methanogens remove excess hydrogen via anaerobic metabolism (Willing, 2011) and, by doing that, favours the growth of fermentative bacteria (Bayané and Guiot, 2010). Acetogenic bacteria

convert H_2 and CO_2 to acetate (Valdez-Vazquez et al., 2005). Acetogenic bacteria and methanogenic archaea bacteria are, however, sensitive to changes in pH (Valdez-Vazquez et al., 2005) and at high pH volatile fatty acids (VFAs) accumulate (Chen, 2007).

Protozoa between 10^3 and 10^5 protozoa per mL have been isolated from the caecum and colon of ponies (Kern et al., 1973). Genera mostly present were *Blepharocorys*, *Buetschlia*, *Cycloposthium* and *Paraisotricha* (Moore and Dehority, 1993). Approximately 72 species, of which most were ciliates, have been isolated from the large intestine and caecum (Frape, 2010). *Blepharoconus benbrooki*, *Cycloposthium* sp., *Paraisotricha minuta* and *Polymorphella ampulla* were isolated from colonic wall tissue (Kirkpatrick and Saik, 1988). Other protozoa that have been isolated from the colon are *Paraisotricha colpoidea*, *Cochliatoxum periachtum*, *Tripalmaria dogieli* and from the caecum *Cycloposthium edentatum* and *Cycloposthium ishikawai* were isolated (Strüder-Kypke et al., 2007). More recently, protozoa of the classes Ciliasida, Litostomatea, Sporozoa and Suctoria were isolated from fresh faeces of horses in Mexico (Güiris et al., 2010). Protozoa assist in the degradation of hemicellulose and pectins and upon removal, dry matter (DM) digestion decreases (Frape, 2010).

Molecular techniques used to determine microbial diversity

By estimation, only 10 to 50% of the intestinal microbiota are culturable mostly due to strict anaerobic growth requirements (Zoetendal et al., 2004). The complete spectrum of microbiota in the equine GIT can thus only be studied by using molecular-based techniques. Techniques most frequently used include 16S rDNA sequencing, non-16S rRNA sequencing, denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), temporal temperature gradient gel electrophoresis (TTGE), terminal-restriction fragment length polymorphism (T-RFLP) analysis, single strand conformation polymorphism (SSCP) analysis, fluorescent *in situ*

hybridization (FISH), dot-blot hybridization, quantitative PCR and a diversity of microarrays (Endo et al., 2009; Morita et al., 2009; Yuki et al., 2000; Zoetendal et al., 2004).

Lactobacillus agilis, *L. crispatus*, *L. reuteri* and *L. salivarius* present in the stratified squamous epithelium of the non-secreting part of the stomach was identified by using DNA-DNA hybridization and 16S rRNA gene sequencing (Yuki et al., 2000). *Lactobacillus salivarius*, *L. mucosae*, *L. delbrueckii* and *Mitsuokella jalaludinii* present in the stomach, caecum, colon and rectum were identified by using RFLP analysis of partially amplified 16S rDNA (Al Jassim et al., 2005). In another study, *Proteobacteria*, *Spirochaetaceae* and *Verrucomicrobiales*, and bacteria belonging to the *Cytophaga-Flexibacter-Bacteroides* and *Clostridium* groups were identified by performing 16S rDNA sequencing on DNA from the caecum, colonic wall tissue, and lumen (Daly et al., 2001).

Studies conducted by using small subunit (SSU) rRNA-targeted oligonucleotide probes have shown that bacteria from an unknown cluster of the Clostridiaceae and Spirochaetaceae groups, the *Cytophaga-Flexibacter-Bacteroides*, and the *Eubacterium rectale-Clostridium coccoides* groups are prevalent in the colon (Daly and Shirazi-Beechey, 2003). Other bacteria that were detected belonged to the *Bacillus-Lactobacillus-Streptococcus* and the *Fibrobacter* groups (Daly and Shirazi-Beechey, 2003). A shift in the microbial population from predominantly Gram-negative to Gram-positive bacteria has been recorded in horses diagnosed with laminitis (Milinovich et al., 2007). *Streptococcus bovis/equinus* were the most prevalent during the onset of laminitis (Milinovich et al., 2007).

Results obtained by PCR-DGGE and 16S rRNA sequencing have shown that *L. equi*, *L. equigenerosi*, *L. hayakitensis*, *L. johnsonii* and *Weissella confusa/cibaria* are the most dominant in the faeces (Morita et al., 2009; Endo et al., 2009). The presence of Bifidobacteria was also detected and identified with nested-PCR to be *Parascardovia denticolens* (Endo et al., 2009). *Streptococcus*

bovis/equinus were the predominant streptococci (Endo et al., 2007). In terms of numbers, the lactic acid bacteria were more dominant than the bifidobacteria (Endo et al., 2007). *Bacteroidales* was also identified in faeces (Dick et al., 2005).

Digestion

Carbohydrates. Starch is fermented to lactic acid by lactobacilli and streptococci in the fundic section of the stomach (Varloud et al., 2007) and further enzymatically degraded in the small intestine to glucose, which is transported across the gastro-intestinal wall (Cunha, 1991; Pilliner, 1993). Residual carbohydrates are fermented in the hindgut, *i.e.* the caecum and colon (Frape, 2010). Lactic acid produced in the small intestine is not well absorbed and is transported to the caecum and colon where it is fermented to propionate (Frape, 2010). The intake of starch has to be carefully controlled, especially in a resting horse, as excessive quantities may increase blood-glucose levels from the normal 4.4-4.7 mmol/L to more than 6.5 mmol/L after 2 h of feeding (Frape, 2010). High levels of glucose can cause colic (Frape, 2010). Celluloses are fermented by bacteria in the caecum and colon to acetate, butyrate and propionate (Cunha, 1991; Frape, 2010). As much as 1.0 g volatile fatty acids are produced per kg body weight (BW) (Elsden et al., 1946). The fatty acids are rapidly absorbed into the bloodstream. Excessive, unabsorbed, levels of fatty acids are detrimental to the gut microbiota (Frape, 2010).

Protein. Proteins are readily degraded to amino acids by proteolytic enzymes in the ileum, but also to some extent by proteolytic bacteria in the large intestine (Frape, 2010). Proteins in the hindgut are not effectively utilized (Cunha, 1991; Frape, 2010; Pilliner, 1993) and only 1-12% of the amino acids are of microbial origin (Frape, 2010). Most of the essential amino acids are thus obtained from plant material (Pilliner, 1993). Amino acids in the large intestine are decarboxylated to amines (Elliott and Bailey, 2006) and excess amino acids are deaminated to urea in the liver (Frape, 2010). Urea is secreted into the ileum and transported to the caecum where bacteria hydrolyse it to ammonia. The ammonia is used for protein synthesis by another group of bacteria (Frape, 2010).

The levels of ammonia are carefully controlled in the liver by converting it to urea. Excessive levels can lead to ammonia toxicity (Frape, 2010).

Fat. A well balanced equine diet consists of only 4% (w/w) fat (Pilliner, 1993). Fat is enzymatically degraded to fatty acids and glycerol in the small intestine and then adsorbed (Cunha, 1991; Frape, 2010). Fatty acids are converted to ATP and acetate in the mitochondria by enzymes of the β -oxidation pathway (Frape, 2010). If effectively digested, fat is an excellent source of energy (Pilliner, 1993).

Vitamins and minerals. Fat soluble vitamins, *i.e.* A (retinol), D (calciferol), K and E (tocopherol), need to be supplied in the diet (Cunha, 1991; Frape, 2010; Pilliner, 1993). Changes in diet intake or metabolism will thus affect the absorption of these vitamins (Otto et al., 1989). Most water-soluble vitamins, *i.e.* vitamins B₁ (thiamine), B₂ (riboflavin), B₃ (niacin), B₅ (pantothenic acid), B₆ (pyridoxine), B₁₂ (cyanocobalamin), B₁₅ (pangamic acid), folic acid, biotin, choline and vitamin C (ascorbic acid) are produced by intestinal microbiota (Cunha, 1991; Frape, 2010; Pilliner, 1993). Some vitamins are produced after chemical changes of a precursor, *e.g.* vitamin A produced from beta-carotene (Pilliner, 1993). Excess vitamins are excreted via urine (Cunha, 1991). The vitamins required are listed in Table 1.

Vitamins play an important role in carbohydrate, fat and protein metabolism and its requirement is directly linked to the fitness level of the animal. An active horse on a high energy diet requires more vitamins (Pilliner, 1993). This is best appreciated if taken into account that the hindgut capacity of a fit horse is less, compared to that of an unfit horse, thus less microbiota available to produce the vitamins required (Pilliner, 1993).

The two groups of essential minerals required are listed in Table 2. Calcium and phosphorus play an important role in bone formation (Frape, 2010; Pilliner, 1993). Calcium is absorbed from the small intestine, while phosphorus is absorbed from the small and large intestine (Cunha, 1991; Pilliner, 1993). Excess phosphorus decreases calcium absorption. It is thus important to keep the

calcium:phosphorus ratio in the 1.6:1 to 2:1 range (Pilliner, 1993). Excess phosphate is secreted into the caecum and ventral colon and reabsorbed within the dorsal and small colon (Frape, 2010).

Magnesium plays a role in calcium and phosphorus metabolism, and serves as an enzyme activator and co-factor in the metabolism of carbohydrates, fats and proteins (Pilliner, 1993). Magnesium is absorbed in the lower part of the small intestine (Frape, 2010) and to a lesser extent in the large intestine (Cunha, 1991).

Grass and hay are good sources of potassium (Pilliner, 1993). Potassium is absorbed just before it reaches the caecum (Frape, 2010) and is associated with acid-base balance, regulation of fluids and carbohydrate metabolism (Pilliner, 1993). Potassium and sodium are important in sugar and amino acid absorption (Pilliner, 1993), functioning of the nervous system and transport of substrates across the cell membrane (Frape, 2010). Reabsorption of sodium takes place within the large intestine (Frape, 2010). Sodium deficiency leads to dehydration (Frape, 2010; Pilliner, 1993) and the insufficient utilization of digested protein (Pilliner, 1993).

Chloride is an important component in bile and hydrochloric acid (Frape, 2010). A chloride deficiency is highly unlikely if the sodium requirements are met (Frape, 2010; Pilliner, 1993). Sulphur in the body is estimated to be 1.5 g/kg BW and is present in the form of amino acids containing sulphur, heparin and water-soluble vitamins (Frape, 2010). Organic sulphur is present in plant protein amino acids, whereas inorganic sulphur makes up about 10-15% of plant sulphur, and is used for protein synthesis by the gut microbiota (Frape, 2010).

Gastro-intestinal related disease and disorders

Acidosis. A diet high in starch increases the number of lactic acid bacteria, especially *Lactobacillus fermentum*, *Lactobacillus delbruekii*, *Lactobacillus mucosae*, *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Streptococcus bovis* (Bailey et al., 2003) and *Streptococcus equines*

(Willing et al., 2009). These organisms produce high levels of lactic acid and volatile fatty acids, specifically propionate (Hintz et al., 1971; Milinovich et al. 2007). The acids accumulate in the stomach (Varlout et al., 2007), caecum and colon (Bailey et al., 2003; de Fombelle et al., 2003; Kern et al., 1973) and reach toxic levels, even for lactate-utilizing bacteria (Clarke et al., 1990).

Lactate is absorbed into the bloodstream and causes lactic acidosis (Al Jassim et al., 2005), also referred to as metabolic acidosis (Garner et al., 1977). One of the symptoms is laminitis, *i.e.* lameness caused by detachment of the distal phalanx and inner hoof wall (Pollitt, 1999). Laminitis may also be caused by an excessive release of endotoxins as Enterobacteriaceae are lysed due to high lactic acid concentrations (Frape, 2010; Garner et al., 1978; Moore et al., 1979). Lactate-utilizing bacteria at this stage are overwhelmed by the influx in lactic acid production and those less tolerant to low pH will also die (Frape, 2010). Horses are prone to contract the disease during hospitalization (Parsons et al., 2007). Lactic acidosis and endotoxaemia may lead to the onset of colic (Frape, 2010; Moore et al., 1981). The amines produced within the digestive system may play a role in the onset of laminitis (Elliott and Bailey, 2006). Respiratory acidosis may also occur, *i.e.* when CO₂ is retained by the lungs and cardiac or peripheral circulation fails (Garner et al., 1977).

Colic. As already mentioned, colic may be caused by increased lactic acid levels. However, parasites, a change in weather conditions or change in diet may also cause colic (Gonçalves et al., 2002; Nieto, 2006). An estimated 30% of all colic cases are impactions of the intestine, mainly of the large intestine (Frape, 2010; Pilliner, 1993). Impactions occur mainly at the pelvic flexure (Frape, 2010; Pilliner, 1993) and the position where the right dorsal colon connects to the small intestine (Frape, 2010). Impaction close to the ileum is especially dangerous, as water from the caecum and ventral colon is poorly reabsorbed, and dehydration and hypovolaemic shock steps in (Frape, 2010). This in turn leads to poor blood circulation.

Spasmodic colic is caused when the muscular wall of the GIT contracts and bowel movement increases (Frape, 2010; Pilliner, 1993). This is usually due to the sudden intake of feed or water too

soon after exercise (Pilliner, 1993), but may also be caused due to a change in diet (Frape, 2010; Meyer, 2001) or due to damage of the gut wall by strongyle larvae (Pilliner, 1993). Symptoms are sweating, mild stress and constant lying down and getting up (Pilliner, 1993). This condition passes relatively quickly and is treated by injection with a muscle relaxer (Frape, 2010; Pilliner, 1993).

The build-up of gas in the intestine, usually caused by an impaction (Frape, 2010; Pilliner, 1993), restricts peristalsis (Frape, 2010) that may lead to fermentation of feed within the stomach and small intestine and, in severe cases, twisting of the intestine and restriction of blood flow (Pilliner, 1993). In this case immediate surgery is required (Frape, 2010; Pilliner, 1993). Typical symptoms are an increased heart and respiratory rate. Alkalosis usually steps in (Frape, 2010). Excessive gas production is often caused by too much cereal in the diet (Frape, 2010). Symptoms are sweating and violent rolling (Frape, 2010; Pilliner, 1993). In some cases it may seem as if the condition has been cured, but if the impaction is not cleared, symptoms will appear within 4-6 h of the next feeding (Frape, 2010).

Grazing in sandy soil areas may cause impaction and chronic inflammation of the intestine (Cunha, 1991; Frape, 2010). The condition may be reversed by increasing the hay intake (Lieb and Weise, 1999; Weise and Lieb, 2001) and daily intake of prebiotics, probiotics and psyllium (Landes et al., 2008).

Pathogens. *Clostridium difficile* is associated with the onset of colic (Arroyo et al., 2007; Båverud et al., 1998; Båverud et al., 2003; Madewell et al., 1995), but has also been isolated from foals with diarrhoea (Jones et al., 1987; Magdesian et al., 2002). The organism is extremely rigid and may survive for four years in the faeces (Båverud et al., 2003).

Clostridium perfringens causes enterocolitis in neonatal foals (Albini et al., 2008; Madewell et al., 1995; Weese et al., 2001). The species secretes endotoxins that, when produced in high concentrations, causes severe damage to the mucosa and ultimately diarrhoea (Frape, 2010).

Corynebacterium pseudotuberculosis, *Rhodococcus equi* and *Streptococcus* spp. cause the formation of abscesses in the abdomen. *Bacillus* spp., *Bacteroides* spp., *Clostridium* spp., Enterobacteriaceae, *Streptococcus* spp., *Staphylococcus* spp. and *Rhodococcus* spp. cause peritonitis. *Salmonella* spp. causes salmonellosis (Guardabassi et al., 2008) and is often responsible for fatal colic (Frape, 2010).

Increased levels of Enterobacteriaceae may lead to endotoxaemia (Frape, 2010). High yeast levels cause colic and gas build-up (Frape, 2010). Feed concentrates should be low in gluten (not too sticky). Hay and straw should not be cut too short, as all of these factors contribute towards the onset of colic (Frape, 2010).

Parasites. Horses become infected with eggs of tapeworms when they feed on hay invested with mites (Frape, 2010; Pilliner, 1993). *Anoplocephala perfoliata* is the most common and is present in 20-80% of horses (Frape, 2010; Proudman, 2003), but *Anoplocephala magna* and *Anoplocephaloides mamillana* have also been recorded (Proudman, 2003). *Anoplocephala perfoliata* attaches to the wall of the ileocaecal junction (Frape, 2010; Pilliner, 1993). Symptoms are spasmodic colic, caused by impaction of the ileum (Pilliner, 1993; Proudman et al., 1998; Trotz-Williams et al., 2008).

Antimicrobials

Classification and mechanism of action. Antimicrobial agents are classified according to their mode of action and are divided into four categories, *i.e.* those that disrupt cell wall synthesis, inhibit protein synthesis, prevent nucleic acid synthesis and inhibit metabolism (Tenover, 2006). Antibiotics that disrupt cell wall synthesis are β -lactams, *e.g.* cephalosporin and penicillin, and glycopeptides of which vancomycin is the best known (Neu, 1992). Examples of antibiotics that inhibit protein synthesis are aminoglycosides, chloramphenicol and tetracyclines (Neu, 1992).

Sulfonamides, trimethoprim and rifampin disrupt nucleic acid synthesis (Neu, 1992). Sulfonamides also inhibit metabolic pathways (Neu, 1992).

Antibiotics in veterinary medicine. Antibiotics used in veterinary medicine are all related to, or in some cases identical to, antibiotics used in the treatment of humans (Ungemach et al., 2006). As in the case of human studies, examples of antibiotic resistant strains of *Campylobacter* spp., *Escherichia coli*, *Listeria* spp. and *Salmonella* spp. have been isolated from horses (Mølbak, 2004). Treatment is either prophylactic or therapeutic (Ungemach et al., 2006). Antibiotics are also administered as feed additives to stimulate growth (Johnston, 2001; Wegener, 2003; Wierup, 2000). However, the use of antibiotic growth promoters (AGPs) has recently been banned (Wegener, 2003). Antibiotics used in the treatment of horses are listed in Table 3.

Prolonged treatment with antibiotics causes an imbalance in the microbiota naturally present in the GIT (Båverud et al., 1997; Båverud et al., 2003; Grønvold et al., 2010; Guardabassi et al., 2008; Gustafsson et al., 1997). Chronic diarrhoea has been connected to the excessive use of the antibiotic oxytetracycline (Frape, 2010). Oxytetracycline has also been connected to the onset of salmonellosis (Frape, 2010). Vancomycin A-resistant enterococci have been isolated from horse faeces (de Niederhäusern et al., 2007). The use of vancomycin is, however, not recommended (Guardabassi et al., 2008).

Herbal treatment is a more natural alternative (Pilliner, 1993). Bee pollen (propolis) has antifungal activity, garlic is antibacterial, antifungal, antiparasitic and antiviral, and ginger is antibacterial (Williams and Lamprecht, 2008). Herbs may also act as adaptogens, *i.e.* stimulate the immune system (Williams and Lamprecht, 2008) and is an excellent source of nutrients (Pilliner, 1993). Some herbs have antioxidant, antiplatelet, antispasmodic, anti-inflammatory and sedative properties (Pilliner, 1993). A staggering nineteen different types of herbal formulas in Chinese veterinary medicine are used to treat diarrhoea (Xie et al., 1997). An example of successful herbal treatment in horses was the administration of the purple coneflower or *Echinacea angustifolia/purpurea*

extract that enhanced the immune system of the host (O'Neill et al., 2002). However, the use of herbs in the treatment of equine diseases or intestinal disorders has not been that successful.

Probiotics

Probiotics are often used as an alternative to antibiotics (Pilliner, 1993). A probiotic is a live microbial feed supplement that improves the microbial balance within the intestine of the host (Fuller, 1989).

Acid and bile tolerance. In order to colonize within the intestine, probiotics need to survive the passage through the acidic stomach (Pilliner, 1993). Gram-positive lactic acid bacteria (*Lactobacillus*, *Streptococcus* and *Pediococcus*) are well equipped to withstand low pH as they produce lactate and acetate during fermentation of sugars (Frape, 2010). More importantly, these bacteria are able to withstand the lytic action of intestinal lysozyme (Frape, 2010). As the probiotic moves through the equine GIT, it must be able to tolerate bile, a substance which is continually secreted in the small intestine (Frape, 2010). Bile salts emulsify lipids within the bacterial cell membrane (Frape, 2010) and alter fatty acids to make the membrane permeable, ultimately killing the bacteria (Gilliland and Speck, 1977). Some of the reasons why Gram-positive bacteria are able to resist these conditions may be due to the following mechanisms:

Adhesion to epithelium and mucus. Probiotic bacteria adhere to epithelium and mucus in the intestine in order to colonize. Cell surface hydrophobicity, electrostatic interactions, passive and steric forces are all factors that attract the probiotic bacteria and epithelium or mucus to achieve initial contact (Schillinger et al., 2005). Various factors like aggregation substances (Ventura et al., 2002), carbohydrates (Vidal et al., 2002), cell-surface proteins (Roos and Jonsson, 2002), hemagglutinins (Andreu et al., 1995), lipoteichoic acids (Greene and Klaenhammer, 1994) and S-layer proteins (Frece et al., 2005) are involved in the adhesion process. However, adherence to

mucus is hindered by low pH levels thus the chances of adherence are lowered after passage through the acidic stomach (Ouwehand et al., 2001).

Pili play an important role in initial adherence to host tissues, followed by colonization of mucosal surfaces (Hendrickx et al., 2009). Pili are localized cell-surface proteinaceous filaments and the genes encoding enterococcal pili are arranged in operons with at least one sortase gene. Two pilin gene clusters, the biofilm enhancer pili operon (*bee*) and the endocarditis and biofilm associated pili operon (*ebp*), have been described for *E. faecalis*. Four pilin gene clusters have been found in *E. faecium*, and their exact role in pathogenicity is the focus of ongoing research (Hendrickx et al., 2009).

Genome sequencing of *L. rhamnosus* GG has revealed the presence of two pilin gene clusters, *spaCBA* and *spaFED*. The *spaFED* gene cluster was also found in *L. rhamnosus* LC705. No homology was found between these clusters and other bacterial pilin genes, although some similarity was found in the protein sequences of pilin proteins found in *E. faecalis* and *E. faecium* (Kankainen et al., 2009). *spaCBA*, only found in the probiotic *L. rhamnosus* GG, was determined to encode mucus binding pili.

Competitive exclusion of pathogens. Intestinal pathogens must adhere to epithelium and survive within the intestine to be pathogenic to the host (Frape, 2010; Lee et al., 2003). Therefore some probiotic bacteria are able to compete with these pathogens for adhesion (Lee et al., 2003). Lactobacilli in particular are able to compete with pathogens by binding to epithelial adhesion sites, thus preventing pathogens from adhering (Montes and Pugh, 1993). Pathogen exclusion may also result from lactobacilli biofilm formation which protects epithelium cells from pathogen adhesion (Montes and Pugh, 1993). Lactobacilli can even displace some of the natural gut bacteria (Lee et al., 2003). The displacement of the natural lactic acid bacteria in the intestine is a necessity under certain conditions (Frape, 2010).

Production of antimicrobial compounds. Lactic acid bacteria produce various antimicrobial agents from carbohydrate fermentation including diacetyl, various organic acids, CO₂, hydrogen peroxide (Naidu et al., 1999) and low molecular weight proteins (Vandenberg, 1993). Diacetyl (2,3-butanedione) is active against Gram-negative bacteria, moulds and yeast (Jay, 1982). Lactic acid and volatile fatty acids are organic acids produced within the intestine, lowering the pH, and inhibiting various Gram-positive and Gram-negative bacteria (Naidu et al., 1999). Lactic acid bacteria also produce other organic acids *i.e.* acetate and propionate (Ouweland and Vesterlund, 2004). Carbon dioxide, a by-product of fermentation, is able to disrupt cell membranes (Lindgren and Dobrogosz, 1990), with Gram-negative bacteria predominantly affected (Devlieghere and Debevere, 2000). Hydrogen peroxide (H₂O₂) inhibits glycolysis (Carlsson et al., 1983). An example of a low molecular weight protein produced by lactic acid bacteria is reuterin (from *Lactobacillus reuteri*) which is able to inhibit viruses, bacteria, fungi and protozoa (Axelsson et al., 1989). Bacteriocins are antimicrobial peptides produced mainly by Gram-positive bacteria as defence (Hansen et al., 1989) against closely related strains (Klaenhammer, 1993). These peptides have various modes of action (Cleveland et al., 2001).

Stimulation of immune response. Probiotics assist in the stimulation of specific and non-specific immune responses (Parvez et al., 2006). Probiotics may increase cytokine levels and natural killer cell activity, activate macrophages, change systemic T cell balance and increase immunoglobulin levels (Parvez et al., 2006). Within the GIT, probiotics promote the proper functioning of the epithelium barrier (Ivanov and Littman, 2011; Kopp-Hoolihan, 2001) while immunoglobulins like IgA are up-regulated and inflammatory cytokines are down-regulated (Kopp-Hoolihan, 2001). The immune system will react towards microorganisms and antigens within the mucosal layer of the GIT (Kopp-Hoolihan, 2001).

Probiotics for animals. Manufacturers of animal feeds use the term direct-fed microbials (DFM) and not probiotics, as directed by the US Food and Drug Administration (FDA) (Yoon and Stern,

1995). When administered to farm animals, DFM will provide a healthy microbial balance within the intestine, more effective digestion and proper absorption of nutrients (Fuller, 1999). The administration of probiotics to farm animals as a supplement helped with digestion and the successful absorption of the nutrients within the intestine (Abe et al., 1995). Enhanced growth rate of the host is also observed when feed is supplemented with probiotic (Pilliner, 1993; Topliff and Monin, 1990). The administration of probiotics also decreased the risk of disease acquisition because of an increased resistance in these animals (Fuller, 1999; Lema et al., 2001).

Equine probiotics. In horses specifically, potential probiotic lactic acid bacteria (*Lactobacillus equi*, *L. crispatus*, *L. johnsonii*, *L. reuteri* and *L. salivarius*) have been administered and proven to be beneficial to the host with regard to growth promotion and resistance to diarrhoea (Yuyama et al., 2004). Diarrhoea was not prevented in neonatal foals when they were administered with a strain of equine origin, *Lactobacillus pentosus* WE7 (Weese and Rousseau, 2005). Enterococci have also proved to maintain probiotic properties when administered to animals (Simonová et al., 2005). During studies on rabbits, *Enterococcus faecium* successfully colonized within the rabbit intestine and may thus have probiotic potential (Simonová, 2006).

The combination of *L. acidophilus*, *L. casei*, *L. plantarum* and *E. faecium* administered to horses as a probiotic controlled *Salmonella* infection (Ward et al., 2004). Two commercial probiotics were used in another study to treat *Salmonella* faecal shedding in horses with colic (Parraga et al., 1997). The first probiotic containing *Lactobacillus acidophilus*, *L. casei*, *L. plantarum* and *Streptococcus faecium* was administered daily at a concentration 3×10^8 cfu while the second probiotic consisting of *Bifidobacterium longum*, *B. thermophilum*, *Lactobacillus acidophilus* and *Streptococcus faecium* was administered at a concentration 4.1×10^9 cfu. These probiotics had no effect on *Salmonella* faecal shedding (Parraga et al., 1997). Another study by Kim et al. (2001) on the prevention of *Salmonella* faecal shedding with a commercial probiotic (*Lactococcus lactis* and *Enterococcus faecium*), administered at 5×10^9 cfu per day, was unsuccessful. The probiotic also contained yeast

cells that were administered at a concentration of 1×10^8 cfu per day (Kim et al., 2001). Swyers et al. (2008) experimented with the administration of single strains and a combination of strains, but none prevented acidosis. The *Lactobacillus acidophilus* strain was administered individually, while the lactic acid bacterial strain mixture contained *Bifidobacterium bifidum*, *Enterococcus faecium*, *Lactobacillus acidophilus* and *Lactobacillus casei* (Swyers et al., 2008). Incorrect dosaging is one of the main reasons why administration of a probiotic does not always work in the host. The suggested dosage of viable probiotic cells for an average 450 kg horse is between 10^{10} and 10^{11} cfu per day (Weese, 2001). In terms of lactobacilli, administration of adequately high doses every day is very important as this lactic acid bacterium is shed quite easily (Montes and Pugh, 1993).

Live yeast cells have also been used as a supplement to horses. The yeast *Saccharomyces cerevisiae* has been administered and ultimately enhanced fibre digestion (Jouany et al., 2009). Other positive effects that these yeast supplements have include improved digestion of cellulose (Jouany et al., 2008), improved digestion of hemicellulose (Glade and Biesik, 1986) and an increase in the amount of anaerobic bacteria within the digestive system (Medina et al., 2002). Interestingly *S. cerevisiae* cells when administered were higher in concentration within the caecum than in the colon (Jouany et al., 2009). This result correlates with the fact that the effects of *S. cerevisiae* administered by Medina et al. (2002) were observed in higher magnitude within the caecum. Another *Saccharomyces* strain, *S. boulardii*, decreased the time that diarrhoea persisted within horses suffering from enterocolitis (Desrochers et al., 2005). *Lactobacillus rhamnosus* strain GG, of human origin, has been orally administered to horses (Weese et al., 2003). The strain, however, did not colonize successfully within the intestine except when high doses were administered (Weese et al., 2003). Possible explanations for the inability of this strain to act as an equine probiotic is because it is of human origin and can therefore not attach properly to the epithelium cells. Competition between the microflora of the horse and the *Lactobacillus* strain could also be a problem as this strain might not be adequately equipped to compete in this environment (Weese et al., 2003).

Probiotics have both negative and positive attributes. When the right combination of strains are chosen, administered at the appropriate concentration and evaluated properly for probiotic potential, the host will benefit from the administration of a probiotic.

Gene transfer amongst intestinal bacteria

Bacteria are known to transfer genetic material through the exchange of bacteriophages, plasmids, transposons and other mobile genetic elements (Van Reenen and Dicks, 2011). This has been shown for intestinal bacteria such as *Campylobacter*, *Haemophilus*, *Helicobacter*, *Neisseria*, *Pseudomonas*, *Staphylococcus* and *Streptococcus* spp. (Thomas and Nielsen, 2005).

Enterococci are often associated with nosocomial infections (Vankerckhoven et al., 2008). Six vancomycin resistance types have been described amongst enterococci (Courvalin, 2006). Resistance to VanC has been reported for *Enterococcus gallinarum* and *Enterococcus casseliflavus-flavescens*. Operons encoding VanA and VanB are located on plasmids or the genome, while operons encoding VanC, VanD, VanE and VanG are located on the chromosome. Resistance to VanA and VanB may be acquired by the transfer of mobile elements (transposons) Tn1546 (VanA) and Tn1547 or Tn1549 (VanB) (Teuber et al., 1999). Launay et al. (2006) reported the transfer of transposon Tn1549 containing the vancomycin B2 operon from *Clostridium symbiosum* to *E. faecium* and *E. faecalis* in the GIT of gnotobiotic mice. Similarly, the transfer of conjugative transposon Tn1545 with a tetracycline resistance gene from *E. faecalis* to *Listeria monocytogenes* was observed (Doucet-Populaire et al., 1991). Bahl et al. studied the *in vivo* transfer of Tn916 conferring tetracycline resistance among strains of *E. faecalis* (Bahl et al., 2004). While transfer took place, some tetracycline sensitive strains also persisted in the intestines of gnotobiotic rats.

Conjugative transposons may also confer resistance to tetracycline, erythromycin, chloramphenicol and kanamycin (Mathur and Singh, 2005). They are often inserted into plasmids or chromosomal genes as single or multiple copies (Mathur and Singh, 2005). Vancomycin A (*vanA*) resistance could be transferred from animals to humans by *Enterococcus faecium* (Bourgeois-Nicolaos et al., 2006), or from porcine to human by enterococci in the gastrointestinal tract (Moubareck et al., 2003). VanA resistance could be transferred from *E. faecium* to *L. acidophilus* *in vivo* (Mater et al., 2008). Apart from this study, few virulence factors have been reported for *Lactobacillus* spp. and *Bifidobacterium* spp. A possible virulence trait in lactobacilli may be the ability to aggregate human platelets, which have been found in strains of *Lactobacillus rhamnosus*, *Lactobacillus paracasei* subsp. *paracasei*, *L. acidophilus*, *L. fermentum*, *L. oris*, *L. plantarum* and *L. salivarius* (Harty et al., 1994).

Streptococcus spp., *Staphylococcus* spp., *Peptostreptococcus* spp., *Propionibacterium* spp. and *Clostridium* spp. produce hyaluronidase (Girish and Kemparaju, 2007; Hynes and Walton, 2000), which degrades hyaluronan to disaccharides that may be transported and metabolized intracellularly by pathogens (Hynes and Walton, 2000; Pecharki et al., 2008; Starr and Engleberg, 2006). The enzyme facilitates the spread of bacteria and toxins through tissue and causes tissue damage (Kayaoglu and Ørstavik, 2004). This may explain why species from these genera cause mucosal or skin infections (Hynes and Walton, 2000; Kayaoglu and Ørstavik, 2004; Pecharki et al., 2008).

Enterococcus faecium and *E. faecalis* produce adhesins Ace and Acm, respectively, which binds to collagen. The two proteins share some similarity on amino acid level (Hall et al., 2007). In human studies, Ace was expressed during infections, whereas Acm was only expressed by clinical isolates of *E. faecium* (Franz and Holzapfel, 2004). Expression of adhesin-like endocarditis antigens by *E. faecalis* are induced when the cells are grown in serum (Franz and Holzapfel, 2004). The antigen SagA, which is essential for *E. faecium* growth, also binds to fibrinogen, collagens, fibronectin, and laminin (Teng et al., 2003).

Intestinal infections are often treated with antibiotics. As in the case of humans and all other animals, administration of antibiotics over an extended period may lead to the development of resistant microorganisms (Wierup, 2000). In light of this, many horse owners have converted to herbal treatments (Pilliner, 1993). Administration of beneficial microorganisms (probiotics) on a regular basis keeps the microbiota in the GIT in balance and has proven to be an effective method of precaution against intestinal infections (Fuller, 1989). Lactic acid bacteria have been used as probiotics in humans and various animals. A number of commercial products containing lactic acid bacteria are available. However, little research has been done on lactic acid bacteria in horses and selection of strains with probiotic properties. In fact, little is known about the interaction between microorganisms in the equine GIT and even less is available on the interaction between microorganisms and intestinal cells.

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Table 1. Required vitamin supplements by the horse (Adapted from Pilliner, 1993)

Water-soluble vitamins	Unit per kg	Total required	Supplement required
Vitamin B ₁ (thiamine)	mg/kg	15	2
Vitamin B ₂ (riboflavin)	mg/kg	15	3
Vitamin B ₃ (niacin)	mg/kg	25	20
Vitamin B ₅ (pantothenic acid)	mg/kg	15	10
Vitamin B ₆ (pyridoxine)	mg/kg	10	2
Vitamin B ₁₂	µg/kg	250	20
Folic acid	mg/kg	10	1
Biotin	µg/kg	200	100
Choline	mg/kg	200	100
Vitamin C	mg/kg	250	100
Fat-soluble vitamins			
Vitamin A	iu/kg (0.3 µg/kg)	12 000	12 000
Vitamin D ₃	iu/kg (0.025 µg/kg)	1200	1200
Vitamin E	mg/kg	200	100

Table 2. Essential minerals required by a horse (Adapted from Pilliner, 1993)

Major minerals	Weight	Per kg of feed	Per day (16 hrs) per 500 kg BW
Magnesium	g	0.9	11
Potassium	g	4.0	50
Sodium	g	3.5	44
Sulphur	g	1.5	19
Trace elements			
Copper	mg	20	250
Zinc	mg	60	750
Manganese	mg	50	625
Iron	mg	150	1900
Iodine	mg	0.15	1.9
Selenium	mg	0.2	2.5
Cobalt	mg	0.2	2.5

Table 3. Antimicrobial treatment for equine gastro-intestinal disease (Adapted from Guardabassi et al., 2008)

Pathogen	Disease	Treatment
<i>Bacillus, Bacteroides,</i>	Peritonitis	Penicillin
<i>Clostridium,</i>		
Enterobacteriaceae,		
<i>Rhodococcus,</i>		
<i>Staphylococcus/Streptococcus</i>		
<i>Clostridium difficile</i>	Diarrhoea	Metronidazole
	Proximal enteritis	
<i>Clostridium perfringens</i>	Diarrhoea	Metronidazole
<i>Corynebacterium pseudotuberculosis,</i>	Abdominal abscess	Trimethoprim/sulphonamides
<i>Rhodococcus equi,</i>		
<i>Streptococcus</i>		
<i>Lawsonia intracellularis</i>	Proliferative enteropathy	Erythromycin
<i>Neorickettsia risticii</i>	Potomac Horse Fever	Oxytetracycline
<i>Salmonella</i>	Salmonellosis	Ampicillin
		Aminoglycoside
		Trimethoprim/sulphonamides

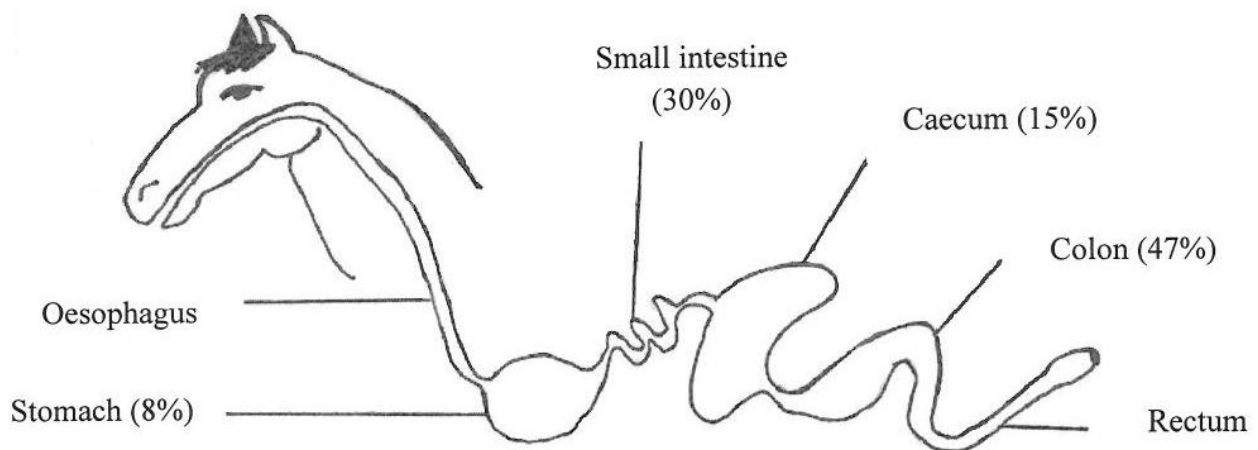


Fig. 1. Schematic presentation of the equine gastro-intestinal tract (Adapted from Cunha, 1991).

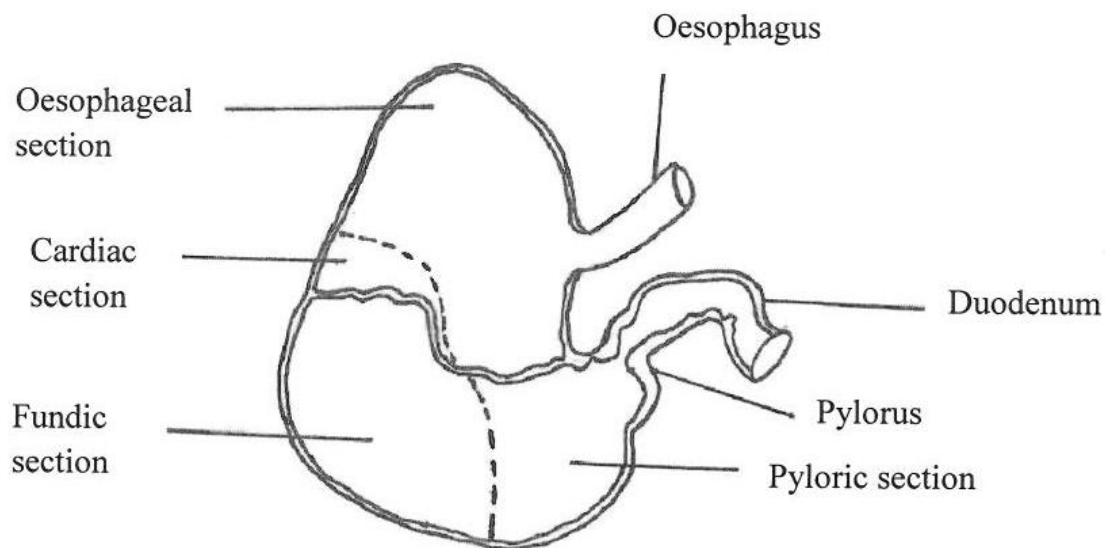


Fig. 2. Anatomy of the stomach (Adapted from Pilliner, 1993).

CHAPTER 3

Probiotic potential of *Lactobacillus equigenerosi*, *Lactobacillus reuteri*, *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA in horses

Written according to *Applied and Environmental Microbiology*

Probiotic Potential of *Lactobacillus equigenerosi*, *Lactobacillus reuteri*, *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA in Horses

MARLIE BOTHA¹, MARELIZE BOTES¹, BEN LOOS² AND LEON M.T. DICKS¹

Department of Microbiology¹ and Department of Physiological Sciences², University of Stellenbosch, Stellenbosch 7600, South Africa.

A combination of *Lactobacillus equigenerosi* Le1, *Lactobacillus reuteri* Lr1, *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA were evaluated as a potential probiotic for horses. Survival of the strains after passage through the gastro-intestinal tract (GIT) and their ability to adhere to epithelial cells were studied. All of the strains, in particular *L. reuteri* Lr1, survived low pH levels (pH 3). Bile salt concentrations of 0.5%, 1.0% and 1.5% (w/v) were tolerated and none of the strains harboured virulence genes such as adhesion to collagen (Ace), aggregation substance (AS), cytolysin (Cyl), non-cytolysin (β hemolysin III) and vancomycin A, B and C. All strains adhered to buccal epithelial cells. However, *L. equigenerosi* Le1 and *E. mundtii* ST4SA invaded the cells. *L. equigenerosi* Le1 competed with *Clostridium* sp. C6 for adhesion to epithelium and co-aggregated with the pathogen. High levels of co-aggregation were recorded between *L. reuteri* Lr1 and *Clostridium* sp. C6.

Lactic acid bacteria (LAB) are often used as probiotics, with the main aim of improving the microbial balance within the intestine of the host (11). Species of *Lactobacillus*, *Enterococcus* and *Bifidobacterium* spp. are usually selected (12). Survival at low pH (1 and 3) and tolerance to bile salts (0.3%, w/v) are some of the most important criteria (19). Tolerance to bile salts is often associated with the presence of bile salt hydrolase (BSH), but this is debateable (27). Adherence to epithelial cells and mucus is also considered an important characteristic, especially when cells compete for colonization (12, 13, 32, 14, 17). Direct contact with epithelial cells may induce an immune response and thus a natural defence against pathogens (26). Production of antimicrobial compounds, for example bacteriocins, is another defence mechanism used by many LAB naturally present in the GIT (15, 21). All probiotic strains should, however, be screened for the presence of virulence genes (23).

Evaluation of strains for probiotic properties is often done *in vitro* (12). Cell lines prepared from the colon or other sections of the GIT are often used to study the interactions of probiotic strains (37). Buccal or cheek epithelial cells have also been used (7, 38, 39). Little research has been published on the equine intestinal epithelium. Yuki et al. (40) studied the adhesion of lactobacilli to the epithelial cells and techniques such as Giemsa staining (8), fluorescent staining (10) Gram-staining (34) and radioactive labelling were used to study microbial interactions. Fluorescent *in situ* hybridization (FISH) has been used to monitor changes of microbial populations in the hindgut of horses diagnosed with laminitis (22).

The aim of this study was to evaluate the probiotic potential of a combination of *Lactobacillus equigenerosi* Le1, *Lactobacillus reuteri* Lr1, *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA. Characteristics studied included survival in the presence of low pH and, bile salts, the ability to adhere to epithelial cells, competition between bacteria for adherence, and virulence based on the presence of encoding genes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Lactobacillus equigenerosi* Le1 and *Lactobacillus reuteri* Lr1 were isolated from horse faeces and *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA from sorghum beer (35) and soy beans (16), respectively. All strains were, grown in MRS broth (Biolab Diagnostics, Midrand, South Africa) at 37°C. Plates streaked with *L. equigenerosi* Le1 and *L. reuteri* Lr1 were incubated at 37°C for 48-72 h in anaerobic flasks containing gas generating envelopes (Anaerobic system BR0038B, Oxoid Ltd., Basingstoke, Hants,

UK). *Clostridium* sp. C6, isolated from faecal samples of a horse with diarrhoea, was grown in Differential Reinforced Clostridial Medium (DRCM) agar (Merck, Darmstadt, Germany). Plates were incubated anaerobically at 37°C for 48-72 h.

pH and bile salt tolerance. The four LAB strains were each cultured in MRS broth (Biolab) for 18 h at 37°C and then inoculated (100 µl) into 10 ml MRS broth (Biolab) pre-adjusted with either 1 M HCl or concentrated DL-lactic acid, to pH 2, 2.6, 3, 4, 5, and 5.4, respectively. The pH of the medium was determined after autoclaving and readjusted if necessary. Samples were taken from the cultures at specific time points, serially diluted in 0.8% (w/v) NaCl and plated out onto MRS agar (Biolab). The same procedure was followed with MRS broth (Biolab) that has been supplemented with 0.5 %, 1.0 % and 1.5 % (w/v) Oxbile (Oxoid). Bile salt hydrolase (BSH) activity was determined by streaking the strains onto MRS (Biolab) agar, supplemented with 0.37 g CaCl₂ and 0.5% (w/v) taurodeoxycholic acid (Sigma). The plates were incubated at 37°C for 24 h and BSH activity recorded positive if a precipitate formed around the colonies.

Virulence genes. The four LAB strains were cultured in MRS broth (Biolab) for 18 h. Genomic DNA was isolated using the ZR Fungal/Bacterial DNA kit (Zymo Research, CA, USA). Plasmid DNA was isolated using the Qiagen plasmid midi kit (Qiagen, Inc., Valencia, USA). Genomic and plasmid DNA were amplified using the primers and conditions listed in Table 1.

Gelatinase activity was tested by streaking active growing cells onto MRS (Biolab) agar supplemented with 3% (w/v) gelatin (BDH Laboratory Supplies, Poole, England). After 24 h of incubation at 37°C, the plates were stored at 4°C for 5 h and examined for opaque zones surrounding the colonies.

Adhesion to epithelium. Buccal (cheek) epithelial cells were sampled from horses. All attempts to culture the epithelial cells failed and experiments had to be performed on samples that have been freshly collected. The swabs were immediately suspended in Minimal Essential Medium (MEM) Earle's Base (Highveld Biological PTY LTD, Kelvin, Johannesburg, SA). An eight-chamber slide (Nalge Nunc International, Rochester, NY, USA) was seeded with 200 µl epithelium cells per well. LAB cells were prepared by suspending 200 µl of each strain ($OD_{600nm} = 1.5$) into 1 ml sterile distilled water. The cells were harvested (10,000 g, 5 min, 18°C) and each strain resuspended in 1 ml sterile PBS. To 1 ml cell suspension, 1.5 ml SYTO 9 (3.34 mM stock solution) and 1.5 ml propidium iodide (20 mM stock solution; LIVE/DEAD BacLight bacterial viability kit, L34856) were added and incubated for 15 min at 25°C. The epithelium-seeded wells were each inoculated with 150 µl ($\pm 1 \times 10^4$ CFU/ml) of a specific bacterial suspension. After 2 h of incubation at 37°C, the bacterial cells that did not adhere to the epithelial cells were withdrawn, plated out onto MRS

(Biolab) agar and incubated at 37°C for 24 h. The same experiment was performed with non-viable epithelium cells.

Images of the bacteria were recorded using an Olympus Cell[^]R system, attached to an IX-81 inverted fluorescence microscope equipped with a F-view-II cooled CCD camera (Soft Imaging Systems). Cells were excited by exposing to 472 nm and 572 nm, respectively, using a Xenon-Arc burner (Olympus Biosystems GMBH). Emission was collected using a UBG triple-bandpass emission filter cube. For the z-stack image frame acquisition, an Olympus UPlan Apo N 100x Oil objective and the Cell[^]R imaging software was used. Images were processed and background subtracted using the Cell[^]R software. Three dimensional images were obtained using the cell[^]R software with a step width of 0.26 μ m represented in fence view and either a 60x (Olympus PlanApo) or 100x (UPlanApo N) oil immersion objective. Specific conditions were maintained for z stack analysis. Parameters for image acquisition were defined and maintained for all experiments. Z-stacks were processed and displayed in the slice view mode, using the Cell[^]R software. For the time lapse series, images were acquired over 20 min with a cycle time of 5 s. Adherence of the bacteria to epithelial cells was expressed as a percentage value, calculated by using the equation % Adhesion = $[(\text{CFU}/\text{ml}_{120}) / (\text{CFU}/\text{ml}_0)] \times 100$ (33). CFU/ml₀ refers to the initial number of bacteria and CFU/ml₁₂₀ to the number of bacteria that adhered to the epithelial cells after 2 h.

Adhesion of pre-treated LAB to epithelium. The four LAB strains were grown in MRS broth (Biolab), for 18 h, harvested (10,000 g, 5 min, 18°C) and washed with 0.8 ml sterile PBS. Cells were then re-suspended in 500 μ l pronase (5.0 mg/ml; Roche Diagnostics GmbH, Mannheim, Germany) or pepsin (5.0 mg/ml; Roche), prepared in sterile PBS. After 1 h at 37°C, the cells were harvested (14,000 g, 10 min, 4°C), washed using two volumes 0.8 ml sterile PBS and re-suspended in 1 ml sterile PBS. Adhesion experiments were performed as described before.

Competition with *Clostridium* sp. for adherence to epithelial cells. To determine if LAB are able to compete with *Clostridium* to adhere to epithelium cells, the adhesion experiment described before was performed. In this case, the epithelium-seeded wells were inoculated with a combination of 100 μ l of a specific LAB strain and 100 μ l of *Clostridium* sp. C6. The percentage adhesion after 2 h was determined from the number of colonies that developed after plating onto MRS (Biolab) and DRCM (Merck) agar.

Cell surface hydrophobicity, auto-aggregation and co-aggregation. Hydrophobicity, auto-aggregation and co-aggregation were determined according to the methods described by Botes et al. (2). The LAB were grown in MRS broth (Biolab) for 18 h at 37°C, the cells harvested (10,000 g, 10 min, 4°C) and washed twice with quarter-strength Ringer's solution (1.5 g NaCl, 0.02 g KCl, 0.03 g

CaCl₂ and 0.03 g NaHCO₃). The optical density (OD) of the cell suspension was recorded at 580 nm (reading 1). Each sample received an equal volume of *n*-hexadecane, was mixed for 2 min and left at 26°C for 30 min to separate into two phases. One millilitre of the top phase was carefully extracted and the OD reading recorded at 580 nm (reading 2). The level of hydrophobicity of each strain was expressed as a percentage value, calculated by using the equation of Doyle and Rosenberg (6):

$$\% \text{ Hydrophobicity} = [(OD_{580 \text{ nm}} \text{ reading 1} - OD_{580 \text{ nm}} \text{ reading 2}) / OD_{580 \text{ nm}} \text{ reading 1}] \times 100.$$

Auto-aggregation of each strain was determined by washing the cells, as described before, and re-suspending them in sterile saline (0.8%, w/v, NaCl). The cell density of each suspension was adjusted to 0.3 (measured at 660 nm). One millilitre of the cell suspension was transferred to a sterile 2 ml plastic cuvette and centrifuged for 2 min at 2,000 *g*. The cell density in the supernatant was recorded immediately after centrifugation (OD₆₆₀0) and 60 min later (OD₆₆₀60). The percentage of cells that aggregated was calculated using the equation of Malik et al. (20):

$$\% \text{ Auto-aggregation} = [(OD_{660} 0 - OD_{660} 60) / OD_{660} 0] \times 100.$$

Co-aggregation of LAB and *Clostridium* sp. C6 was determined as follows. The LAB strains were each cultured in MRS broth (Biolab) for 18 h at 37°C. *Clostridium* sp. C6 was cultured in DRCM broth (Merck) for 24 to 48 h at 37°C under anaerobic conditions (anaerobic flasks with gas generating envelopes). Cells were harvested (10,000 *g*, 10 min, 18°C), washed with sterile saline (0.8%, w/v, NaCl) and equal volumes of LAB and *Clostridium* sp. C6 cell suspensions mixed. The cell density of the supernatant was recorded (OD₆₆₀Tot) to represent the initial OD. Cells were then harvested (2,000 *g*, 2 min, 18°C) to determine the OD of the cell-free supernatant (OD₆₆₀S). The supernatant was discarded, the pellet diluted and then plated onto MRS (Biolab) and DRCM (Merck) agar, respectively. Incubation was at 37°C (24 h for MRS plates and 48-72 h for DRCM plates). The percentage co-aggregation was calculated using the equation of Malik et al. (20):

$$\% \text{ Co-aggregation} = [(OD_{660} \text{Tot} - OD_{660} \text{S}) / OD_{660} \text{Tot}] \times 100.$$

RESULTS

Survival of LAB at different pH. Survival of the four LAB strains at different pH values, is shown in Fig. 1. None of the strains survived pH 2.0 set with HCl, with the exception of *L. reuteri* Lr1 (10⁶ CFU/ml). *L. reuteri* Lr1, however did not survive pH 2.0 set with DL-lactic acid. All of the strains seemed to survive pH 2.6 set with HCl, but when the pH was set with DL-lactic acid, *L.*

reuteri Lr1 and *L. plantarum* 423 were the only two strains that survived (10^3 CFU/ml and 10^1 CFU/ml, respectively). At pH 3.0 set with HCl, *L. equigenerosi* Le1 and *L. reuteri* Lr1 yielded 10^6 CFU/ml, and *L. plantarum* 423 and *E. mundtii* ST4SA 10^7 CFU/ml. Results obtained at pH 3.0 set with DL-lactic acid, were that *L. reuteri* Lr1 had the lowest CFU/ml (10^5 CFU/ml), whereas *L. equigenerosi* Le1, *L. plantarum* 423 and *E. mundtii* ST4SA all had 10^6 CFU/ml after 2 h. *L. reuteri* Lr1 at pH 4.0 set with HCl, once again had the lowest cell numbers (10^6 CFU/ml), and all of the other strains had 10^7 CFU/ml. Two strains (*L. equigenerosi* Le1 and *L. reuteri* Lr1) had 10^6 CFU/ml after 2 h of incubation at pH 4.0 set with DL-lactic acid, whereas *L. plantarum* 423 and *E. mundtii* ST4SA had 10^8 CFU/ml after 2 h of incubation. Strains survived pH 5.0 set with either HCl or DL-lactic acid, 10^8 CFU/ml were recorded for all strains, except with *L. equigenerosi* Le1 when the pH was set with DL-lactic acid (10^7 CFU/ml). Strains incubated at pH 5.4 set with either HCl or DL-lactic acid survived, but the cell numbers were less (10^7 CFU/ml) compared to incubation at pH 5.0. *E. mundtii* ST4SA was the exception and had 10^6 CFU/ml after 2 h of incubation at pH 5.4 set with HCl.

Bile salt tolerance. Tolerance to bile salts is shown in Fig. 2. *L. equigenerosi* Le1 and *L. reuteri* Lr1 survived after 2 h in the presence of 0.5% (w/v) bile salts with a cell count of 10^6 CFU/ml, whereas *L. plantarum* 423 and *E. mundtii* ST4SA had 10^8 CFU/ml after 2 h of incubation. Results obtained of growth in the presence of 1.0% and 1.5% (w/v) bile salts were similar to growth in 0.5% (w/v) bile salts. *L. equigenerosi* Le1, *L. reuteri* Lr1, *L. plantarum* 423 and *E. mundtii* ST4SA had no BSH activity.

Virulence genes. No virulence genes were amplified from the DNA of *L. equigenerosi* Le1, *L. reuteri* Lr1 and *L. plantarum* 423. *E. mundtii* ST4SA harboured the following virulence genes: aggregation (AS), cytolysin (Cyl) and non-cytolysin (β hemolysin III). None of the strains had gelatinase activity.

Adhesion to epithelium. Adherence of the four LAB strains to viable buccal epithelial cells is shown in Fig. 3. *L. plantarum* 423 and *E. mundtii* ST4SA adhered stronger to epithelium cells compared to *L. equigenerosi* Le1 and *L. reuteri* Lr1. However, *L. equigenerosi* Le1 and *E. mundtii* ST4SA invaded the cells after 20 min (Fig. 3b). All four LAB strains adhered to non-viable epithelial cells and invaded the cells after 20 min (Fig. 4b). *L. plantarum* 423 cells showed the best adhesion (8%) to viable epithelial cells. On the other hand, only 1% of *L. equigenerosi* Le1 and 3% of *L. reuteri* Lr1 and *E. mundtii* ST4SA cells adhered to the epithelial cells. The best adhesion to non-viable cells was observed with *L. plantarum* 423 and *E. mundtii* ST4SA, whereas *L. equigenerosi* Le1 showed the least adhesion.

Adhesion of pre-treated bacterial cells to epithelium. Adhesion of pronase-treated bacterial cells to epithelial cells is shown in Fig. 5a) and b). Adhesion was at its optimal after 2 h. A few cells of *L. equigenerosi* Le1 and *L. plantarum* 423 were observed inside the cells after 1 h of incubation. *L. reuteri* Lr1 also invaded the epithelial cells after 1 h, but no cells were detected after 2 h of incubation (Fig. 5). The percentage adhesion was significantly higher when compared to adhesion of untreated cells, with *L. equigenerosi* Le1 and *L. reuteri* Lr1 showing the least, and *L. plantarum* 423 and *E. mundtii* ST4SA the most adhesion.

Adhesion of pepsin-treated bacteria is shown in Fig. 6. The best adhesion was recorded after 2 h of incubation. *L. equigenerosi* Le1, *L. plantarum* 423 and *E. mundtii* ST4SA invaded the epithelial cells after 1 h of incubation and remained in the cells (Fig. 6). *E. mundtii* ST4SA adhered the best, followed by *L. reuteri* Lr1, *L. equigenerosi* Le1 and *L. plantarum* 423.

Competition with *Clostridium* sp. C6 for adherence to epithelial cells. Results obtained from the competitive exclusion experiment is shown in Fig. 7. In the presence of *E. muntii* ST4SA, only 1% of *Clostridium* sp. C6 cells adhered to the epithelial cells (Fig. 7). However, in the presence of *L. equigenerosi* Le1, *L. reuteri* Lr1 and *L. plantarum* 423, *Clostridium* sp. C6 adhered to the epithelial cells at 3%, 5% and 7%, respectively (Fig. 7).

Hydrophobicity, auto-aggregation and co-aggregation. *L. plantarum* 423 revealed the highest hydrophobicity (50%), followed by *L. equigenerosi* Le1 (8%). *E. mundtii* ST4SA and *L. reuteri* Lr1 showed no hydrophobicity. High auto-aggregation (60%) was recorded for *L. equigenerosi* Le1, followed by 56% for *E. mundtii* ST4SA, 54% for *L. plantarum* 423 and 22% for *L. reuteri* Lr1. The *Clostridium* sp. C6 had an auto-aggregation percentage of 35%. The highest co-aggregation with *Clostridium* sp. C6 was observed with *L. equigenerosi* Le1 (57%) and *L. reuteri* Lr1 (47%). Only 31% of *E. mundtii* ST4SA and 19% of *L. plantarum* 423 cells co-aggregated with *Clostridium* sp. C6. *Clostridium* 10^8 CFU/ml co-aggregated with 10^8 CFU/ml of *L. equigenerosi* Le1, *L. reuteri* Lr1, *L. plantarum* 423 and *E. mundtii* ST4SA.

DISCUSSION

The pH within the equine stomach ranges between 2.6 and 5.4 (25). Digesta will pass onto the rest of the GIT rapidly, but a portion will remain within the stomach for 2-6 h. The LAB strain that showed the best survival within the lowest gastric pH (pH 2.0) was *L. reuteri* Lr1. However, *L. reuteri* Lr1 did not survive pH 2.0 when DL-lactic acid was used to set the pH, and therefore this strain might not survive when too much lactic acid is produced by other LAB within the stomach.

E. mundtii ST4SA and *L. plantarum* 423 had the second and third best survival, respectively, within pH 2.0 set with HCl. When carbohydrates are fermented, lactic acid production will lower the digesta pH to 2.6 (9). All of the strains can survive pH 2.6 set with HCl. *L. reuteri* Lr1 and *L. plantarum* 423 survived at this pH level when set with DL-lactic acid, but the lactic acid affected *L. equigennerosi* Le1 and *E. mundtii* ST4SA. The four strains were able to survive pH 3-5.4 (set with HCl). pH 3.0 set with DL-lactic acid, slightly decreased cell numbers from 10^7 CFU/ml to 10^6 CFU/ml (*L. plantarum* 423 and *E. mundtii* ST4SA). *L. equigennerosi* Le1 growth at pH 3.0 was not effected when the pH was set with DL-lactic acid. Growth of *L. reuteri* Lr1, however, was effected by lactic acid. Strains thrived from pH 4.0-5.4 set with either HCl or DL-lactic acid. *L. reuteri* Lr1 growth at pH 4.0 set with HCl, was the worst of all the strains at this pH. Lactic acid had an effect on *L. equigennerosi* Le1 and *L. reuteri* Lr1 growth at pH 4.0 as these two strains had 10^6 CFU/ml opposed to the 10^8 CFU/ml of *L. plantarum* 423 and *E. mundtii* ST4SA. Growth of all of the strains at pH 5.0 set with either HCl or DL-lactic acid, was better (10^8 CFU/ml compared to 10^7 CFU/ml) than growth at pH 5.4 set with either HCl or DL-lactic acid. *L. equigennerosi* Le1 was not affected by the pH shift but *E. mundtii* ST4SA was affected when pH 5.4 was set with HCl, as cell numbers decreased from 10^8 CFU/ml to 10^6 CFU/ml. All four selected strains can therefore survive the acid environment created by gastric secretions in order to pass onto the rest of the GIT. Excessive lactic acid secreted during carbohydrate overload (1) might however be lethal for these strains up to pH 3.0. *E. mundtii* ST4SA and *L. plantarum* 423 survived within pH 3.7 in a gastro-intestinal model (GIM) simulating human intestinal conditions (3).

Bile is continuously secreted within the small intestine because of the fact that horses do not have a gall bladder (9). The four selected LAB strains survived bile salt concentrations of 0.5%, 1% and 1.5% (w/v) over a period of 2 h and will therefore most probably survive passage through the equine small intestine. As none of these strains have BSH activity, the tolerance of bile salts cannot directly be attributed to the presence of BSH activity in LAB.

L. equigennerosi Le1 and *L. reuteri* Lr1 do not have any of the virulence genes that were screened for and the presence of gelatinase activity could not be detected. *L. plantarum* 423, when studied by Botes et al. (3), also had none of these virulence genes, whereas *E. mundtii* ST4SA had three of these genes but it was speculated that these genes are not expressed. No gelatinase activity was detected in either of the strains. These two strains have been evaluated for their safety in rats and were successful (29). These four selected LAB strains therefore might be safe as these strains seem to not be potentially virulent.

Lactobacilli in animals adhere better to stratified squamous epithelium cells rather than columnar epithelium cells and mucus when it comes to humans (36). Adhesion potential of LAB to epithelium cells is an important factor to take into consideration when the administration dosage is calculated (2).

The best adhesion to viable buccal epithelium cells was seen with *L. plantarum* 423 (8%). This strain had an adhesion percentage of 8% to Caco-2 cells in a study by Botes et al. (2). *E. mundtii* ST4SA had 3% adhesion to the buccal epithelium whereas adhesion to Caco-2 cells was 6% (2). *L. reuteri* Lr1 also had 3% adhesion to viable epithelium whereas in a study by Yuki et al. (40), *L. reuteri* Lr1 isolated from the equine stomach did not adhere *in vitro* to horse or rat epithelium cells. It was interesting to note that *L. equigenerosi* Le1 and *E. mundtii* ST4SA were the only strains that invaded the epithelium cells. The mechanism of invasion is not fully understood and it is not clear as to why these strains invade these cells as the cells remain viable.

Adhesion of the selected LAB strains to non-viable epithelium was observed, the best adhesion was seen with *E. mundtii* ST4SA and *L. plantarum* 423. *L. reuteri* Lr1 adhesion was triple that of adhesion to viable epithelium (9%). The worst adhesion was again seen with *L. equigenerosi* Le1 that had a mere 4% adhesion. Not surprisingly, all four of the LAB strains invaded the cells as these cells are at the start of cell death with the membrane that starts to become permeable.

LAB strains pre-treated with pronase and pepsin had very good adhesion to epithelium cells. Pronase-treated *L. equigenerosi* Le1, *L. reuteri* Lr1 and *L. plantarum* 423 strains invaded cells after 1 h of incubation but after 2 h, *L. reuteri* was absent from inside the cells. *L. equigenerosi* Le1, *L. plantarum* 423 and *E. mundtii* ST4SA invaded cells after 1 h. The ability of *E. mundtii* ST4SA to invade these epithelium cells was, therefore, affected by the pronase treatment but not by the pepsin treatment. Interestingly, after pronase-treatment, *L. reuteri* Lr1 could invade the cells but only after 1 h of incubation. Pronase- and pepsin-treated *L. plantarum* 423 invaded the epithelium cells. Protein adhesins in the cell membranes of the LAB strains were clearly not affected by these two proteolytic enzymes as adhesion was not affected. When Botes et al. (2) treated *L. plantarum* 423 and *E. mundtii* ST4SA with pepsin, pronase and trypsin, adhesion of both strains to Caco-2 cells were affected.

Competitive exclusion of an equine pathogen (*Clostridium* sp. C6) revealed that *L. equigenerosi* Le1 was able to adhere the best when competing with the *Clostridium* sp. C6. *L. equigenerosi* Le1 had 5.25% adhesion whereas *Clostridium* sp. C6 had 3% adhesion. The *Clostridium* sp. C6 still adhered quite well (3%) but adhered better when competing with *L. plantarum* 423 (7%) and *L. reuteri* Lr1 (5%). Even though *E. mundtii* ST4SA did not adhere well (1%) to the epithelium cells

when competing with the pathogen, *Clostridium* also had bad adhesion (1%). The ability of the strains to invade the epithelium cells was not hindered by the presence of the *Clostridium* sp. C6, it is however not clear if the *Clostridium* sp. C6 also invaded the cells. *L. plantarum* 423 hindered adhesion of *Clostridium sporogenes* LMG 13570 to Caco-2 cells in a study by Ramiah et al. (28).

Cell surface hydrophobicity is an important factor for first contact between bacteria and epithelium cells or mucus (33). Hydrophobicity of *L. plantarum* 423 and *E. mundtii* ST4SA determined by Botes et al. (2) was 50% and 0%, respectively. *L. equigenerosi* Le1 and *L. reuteri* Lr1 have 8% and 0% hydrophobicity, respectively. Hydrophobicity does however not necessarily correlate with the probability of adhesion to epithelium cells (2), as was seen in this study, all of the strains adhered to epithelium cells even though *L. reuteri* Lr1 and *E. mundtii* ST4SA have 0% hydrophobicity.

The potential of the LAB strains to form biofilms in order to prevent pathogen colonization is studied by determining the auto- and co-aggregation of the strains (5). *L. plantarum* 423 and *E. mundtii* ST4SA have good auto-aggregation (2) and *L. equigenerosi* Le1 also seems to be able to aggregate well. Co-aggregation with the *Clostridium* sp. C6 was the best with *L. equigenerosi* Le1 and *L. reuteri* Lr1. *E. mundtii* ST4SA was able to co-aggregate with this pathogen the best compared to *L. plantarum* 423. As *E. mundtii* ST4SA and *L. plantarum* 423 produce bacteriocins, co-aggregation with the pathogen will allow pathogenic growth inhibition as antimicrobial substances are produced in close contact with the pathogen (30).

CONCLUSIONS

In vitro evaluation of the selected LAB strains is important as survival through the GIT, colonization and safety within the host are some of the criteria that needs to be met in order to be administered as a probiotic. All of the selected strains survived low intestinal pH conditions and bile salt concentrations. Lactic acid production within the GIT by other LAB was also tolerated to a degree. Virulence genes were absent in all of the strains and adhesion to buccal epithelium cells were good. The ability to successfully compete with *Clostridium* sp. C6 for adhesion was seen with *L. equigenerosi* Le1. Co-aggregation with the *Clostridium* sp. C6 was the best with *L. equigenerosi* Le1 and *L. reuteri* Lr1. The combination of *L. equigenerosi* Le1, *L. reuteri* Lr1, *L. plantarum* 423 and *E. mundtii* ST4SA therefore can be used as potential probiotics for horses. Future studies have to focus on the ability of these strains to adhere to epithelium cells isolated from the lower part of the equine GIT and *in vivo* studies are necessary.

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TABLE 1. Primers used to screen for presence of virulence and resistance genes (Adapted from 3)

Virulence genes	Primers	Positive control	Reference
Adhesion to collagen (Ace)	f:3'-GAATTGAGCAAAAGTTCAATC-5' r:3'-GTCTGTCTTTTCACTTGTTTC-5'	-	(24)
Aggregation substance (AS)	f:3'-AAGAAAAAGAAGTAGACCACC-5' r:3'-AAACGGCAAGACAAGTAAATA-5'	<i>E. mundtii</i> ST4SA	(24)
Cytolysin (Cyl)	f:3'-TGGGTTGGTGGCGGTATTTT-5' r:3'-TAATGCACCTACTCCTAAGCC-5'	<i>E. mundtii</i> ST4SA	(24)
Non-cytolysin (β hemolysin III)	f:3'-TTCGATCATAGTTCKATTTT-5' r:3'-AACAAATGCCAAACMACATGC-5'	<i>E. mundtii</i> ST4SA	(3)
Vancomycin (vanA)	f:3'-TCTGCAATAGAGATAGCCGC-5' r:3'-TCTGCAATAGAGATAGCCGC-5'	-	(18)
Vancomycin (vanB)	f:3'-GCTCCGCAGCCTGCATGGACA-5' r:3'-ACGATGCCGCCATCCTCCTGC-5'	-	(18)
Vancomycin (vanC1)	f:3'-GAAAGACAAAGGAGGACCGC-5' r:3'-TCGCATCACAAGCACCAATC-5'	-	(4)
Vancomycin (vanC2)	f:3'-CGGGGAAGATGGCAGTAT-5' r:3'-CGCAGGGACGGTATTTT-5'	-	(31)
Vancomycin (vanC3)	f:3'-GCCTTTACTTATTGTTCC-5' r:3'-GCTTGTTCTTTGACCTTA-5'	-	(4)

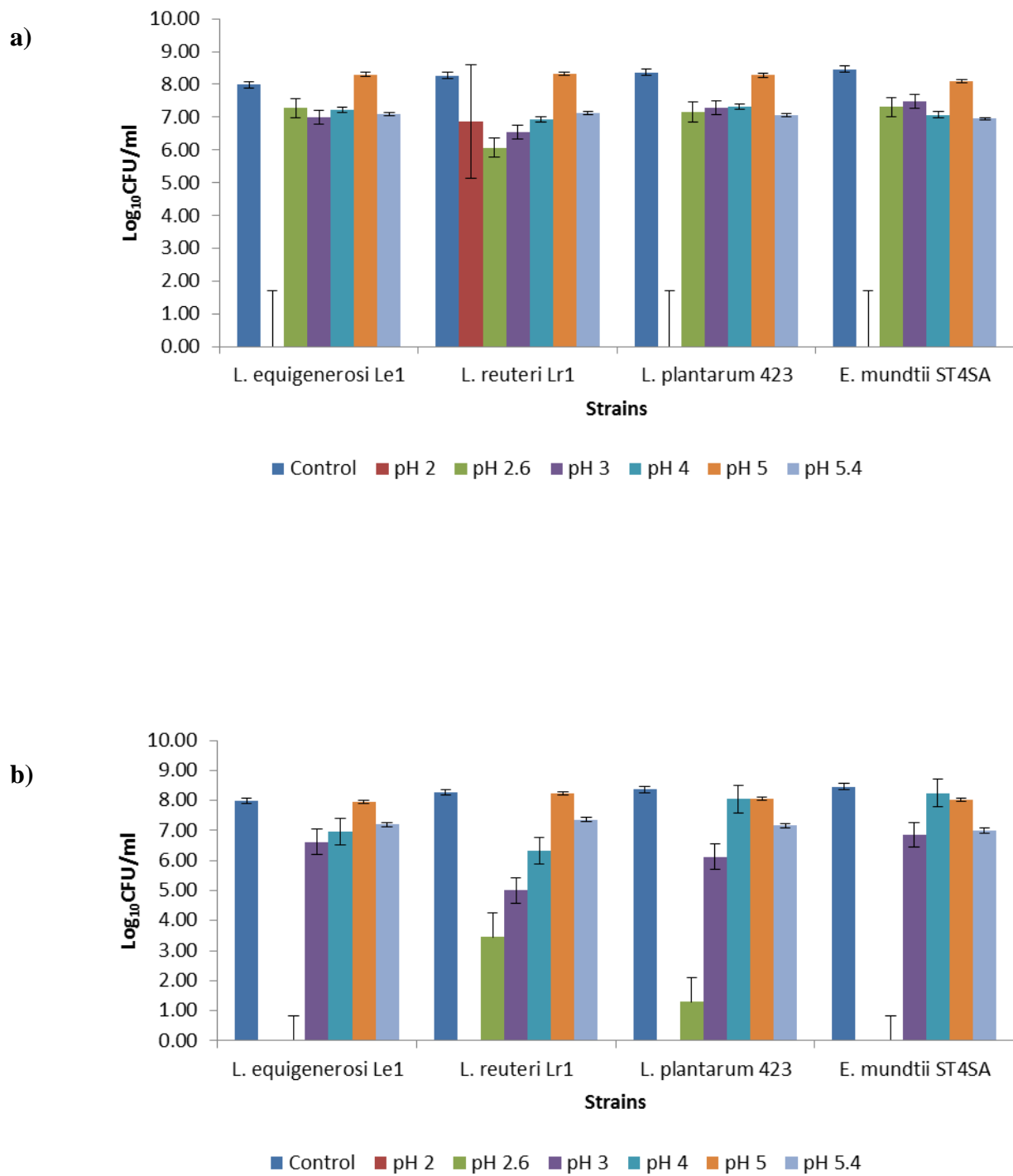


FIG. 1. Survival of LAB strains after 2 h a) pH adjusted with HCl and b) pH adjusted with DL-lactic acid.

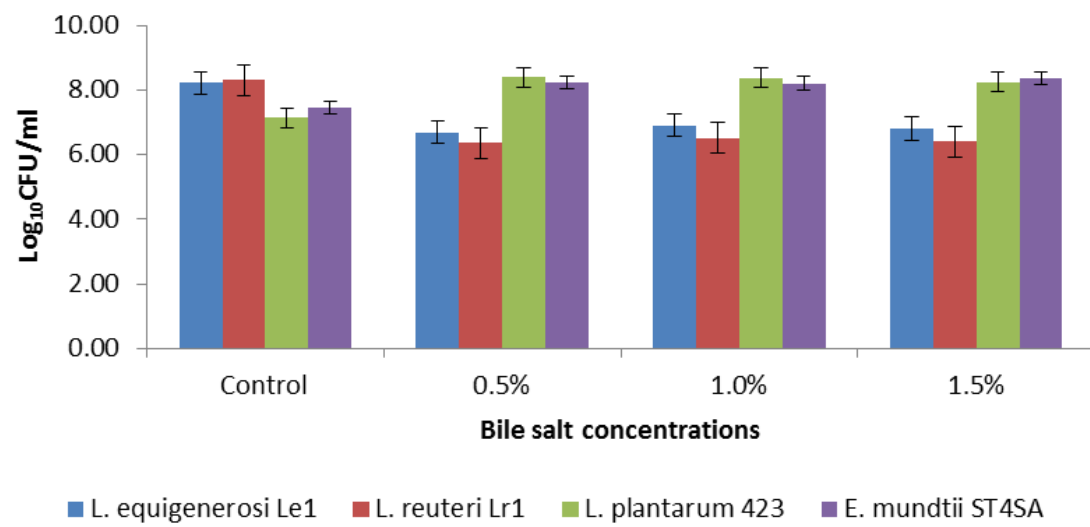


FIG. 2. Survival of LAB strains in bile salts (% w/v) after 2h of incubation.

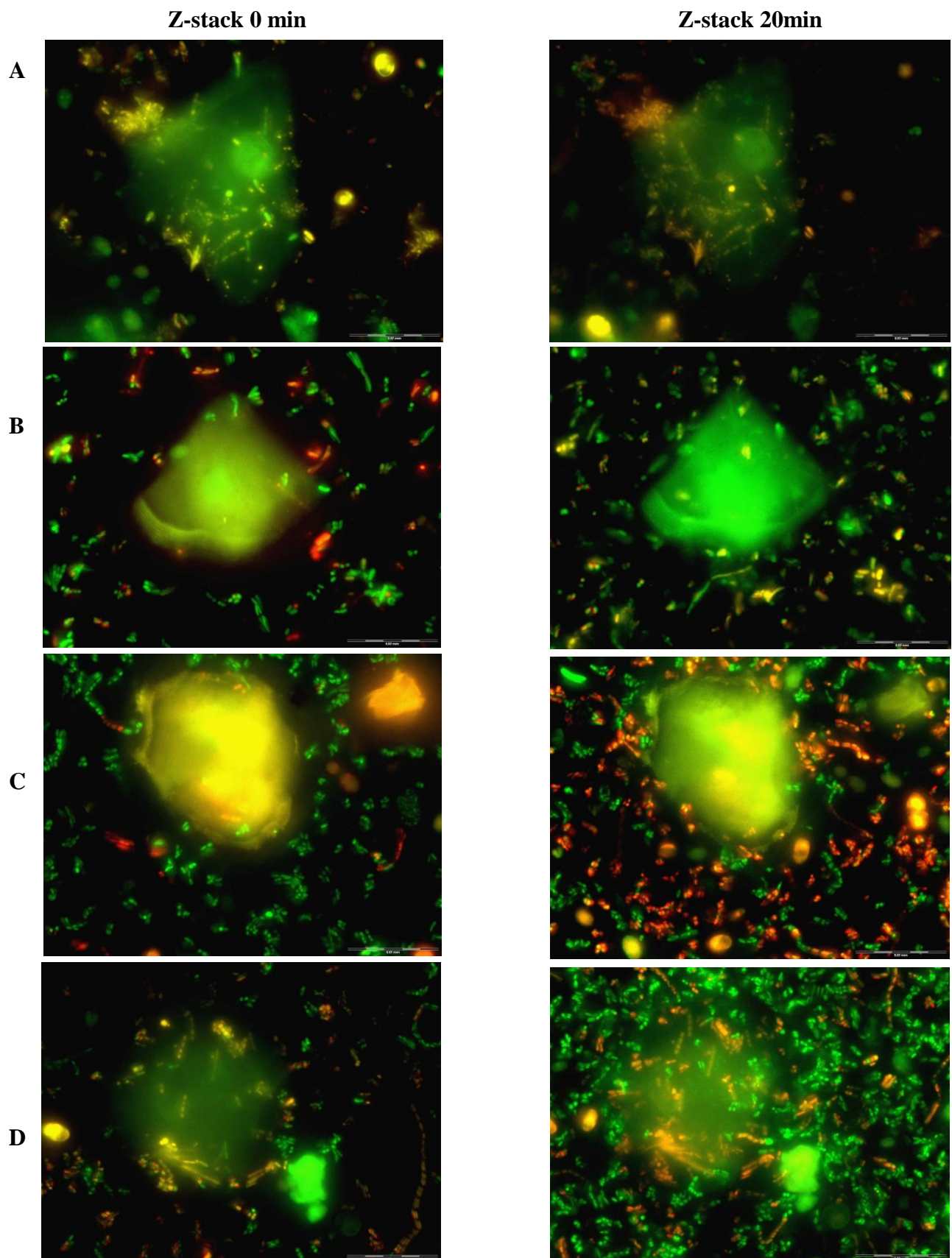


FIG. 3a. Adhesion of **A:** *L. equigenosus* Le1 (1.25%), **B:** *L. reuteri* Lr1 (3.2%), **C:** *L. plantarum* 423 (8.47%) and **D:** *E. mundtii* ST4SA (3.3%) to viable epithelial cells. SYTO 9 stain viable cells green and PI stain membrane-compromised cells red. Epithelial nuclei stained green, indicating viability.

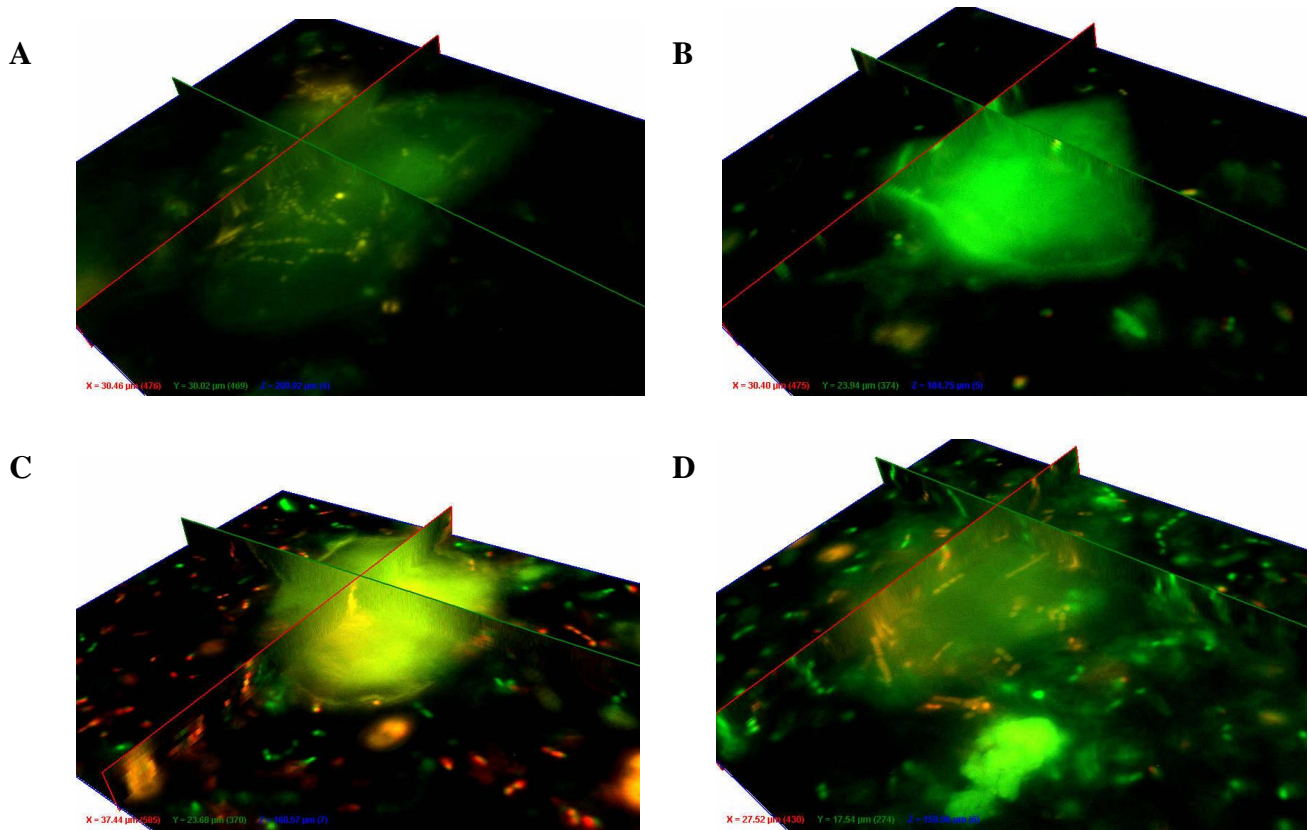


FIG. 3b. Fence view of bacterial strains **A:** *L. equigenerosi* Le1, **B:** *L. reuteri* Lr1, **C:** *L. plantarum* 423 and **D:** *E. mundtii* ST4SA adhering to viable epithelial cells after 20 min.

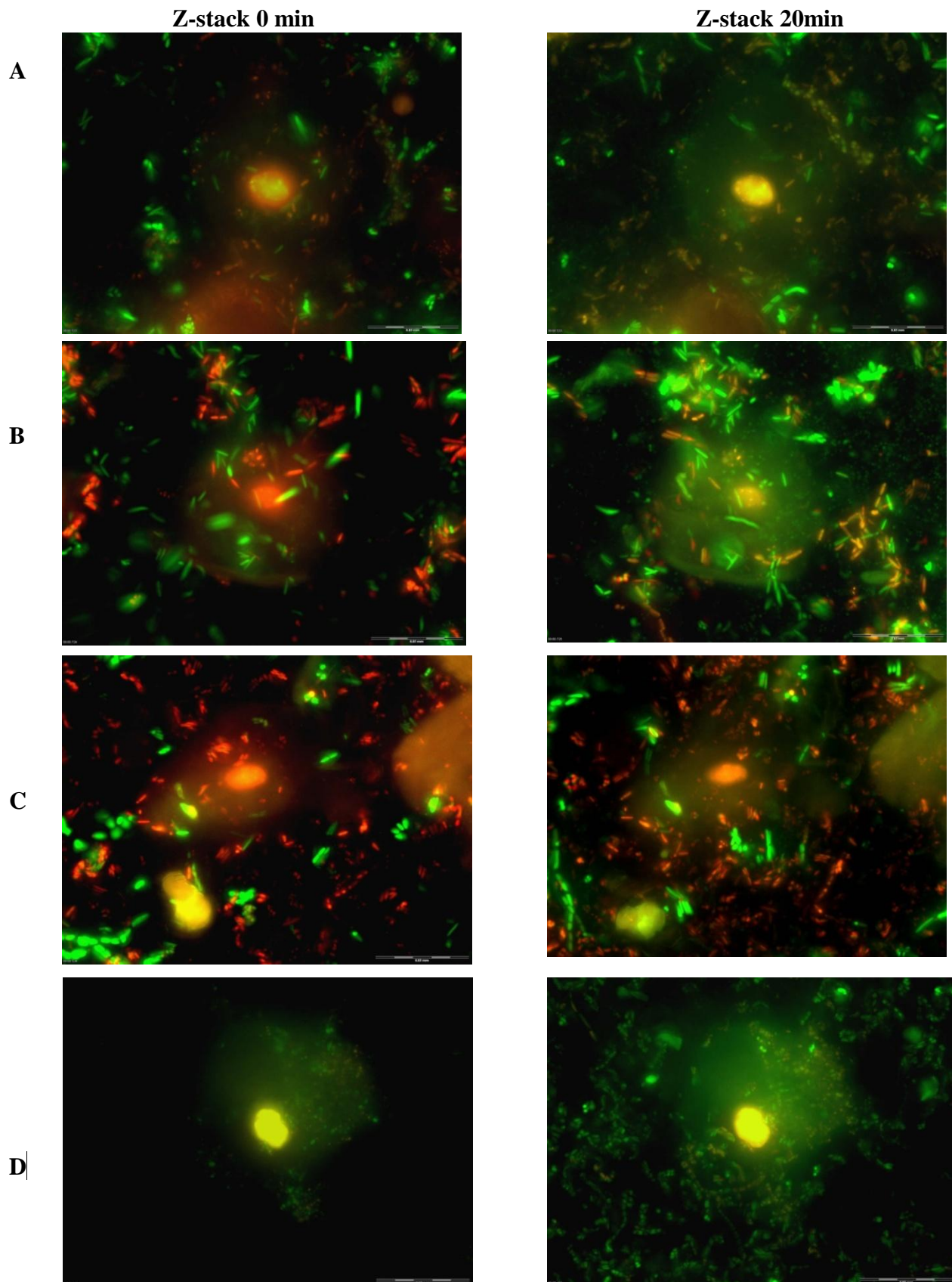


FIG. 4a. Adhesion of bacterial strains **A**: *L. equigennerosi* Le1 (42.5%), **B**: *L. reuteri* Lr1 (9.67%), **C**: *L. plantarum* 423 (36.4%) and **D**: *E. mundtii* ST4SA (100%) to non-viable epithelial cells. SYTO 9 stain viable cells green and PI stain membrane-compromised cells red. Epithelial nuclei stained red, indicating that cells are no longer viable.

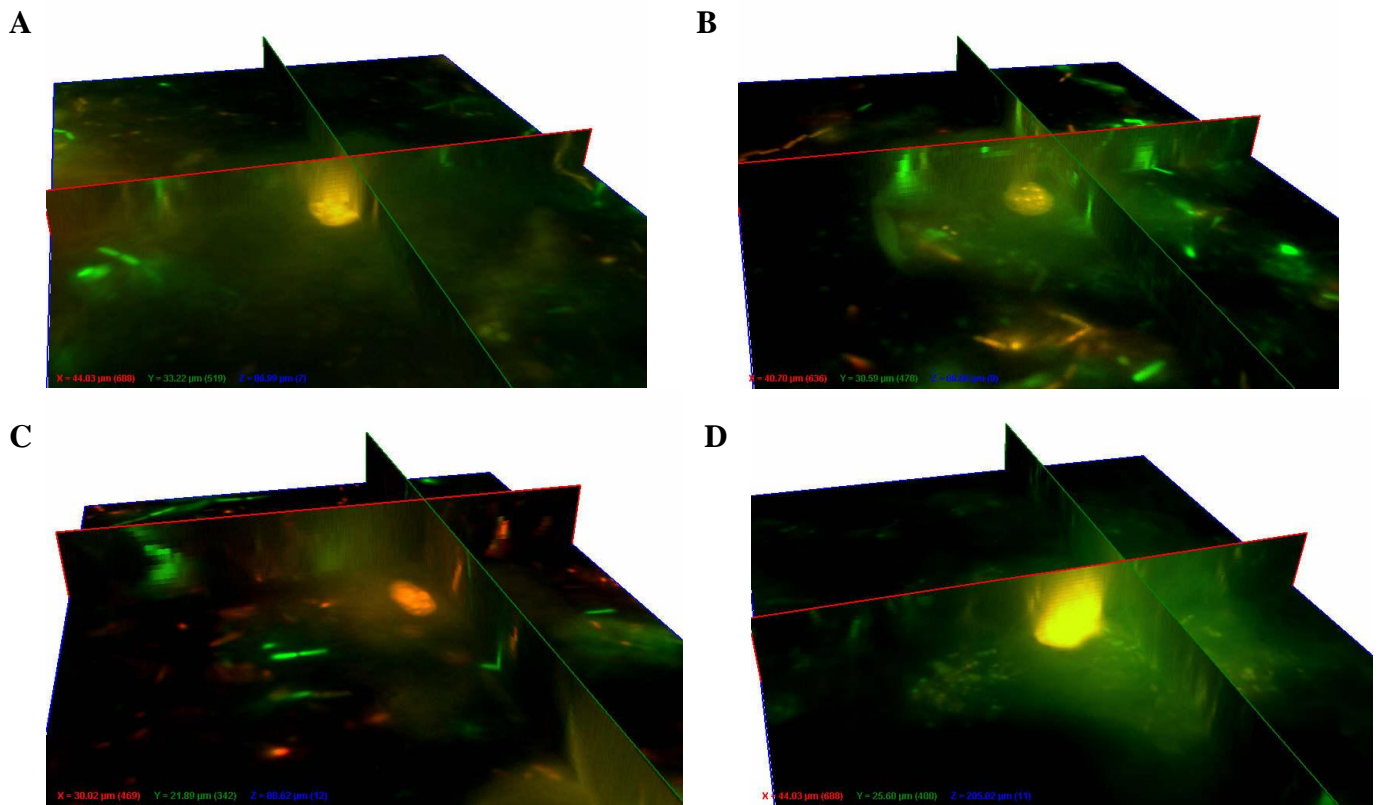


FIG. 4b. Fence view of adhesion of bacterial strains **A**: *L. equigennerosi* Le1, **B**: *L. reuteri* Lr1, **C**: *L. plantarum* 423 and **D**: *E. mundtii* ST4SA to non-viable epithelial cells after 20 min.

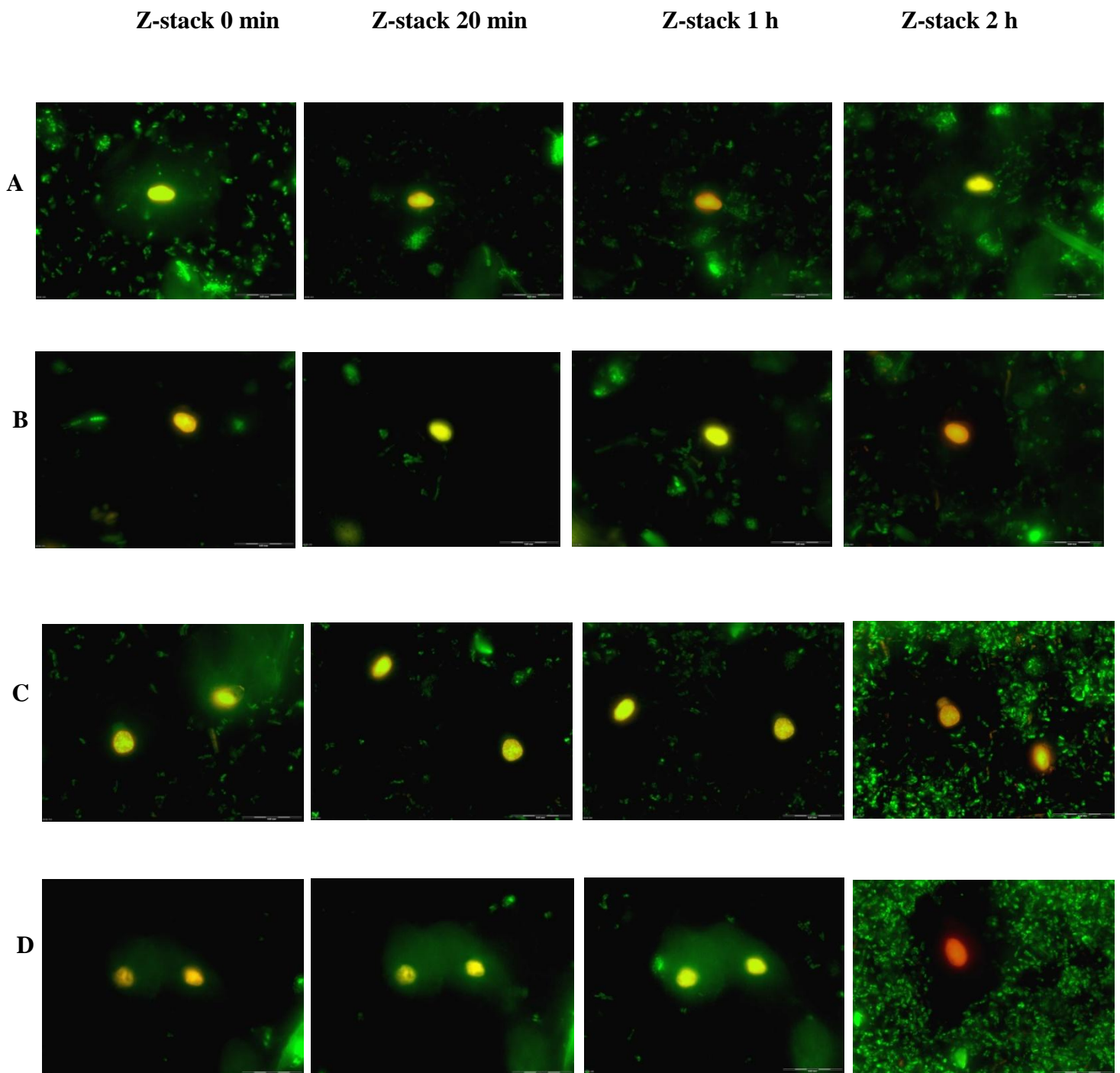


FIG. 5a. Adhesion of bacterial strains **A**: *L. equigenersi* Le1 (43.75%), **B**: *L. reuteri* Lr1 (41.9%), **C**: *L. plantarum* 423 (100%) and **D**: *E. mundtii* ST4SA (100%), pre-treated with pronase, to epithelium at 0 min, 20 min, 1 h and 2 h.

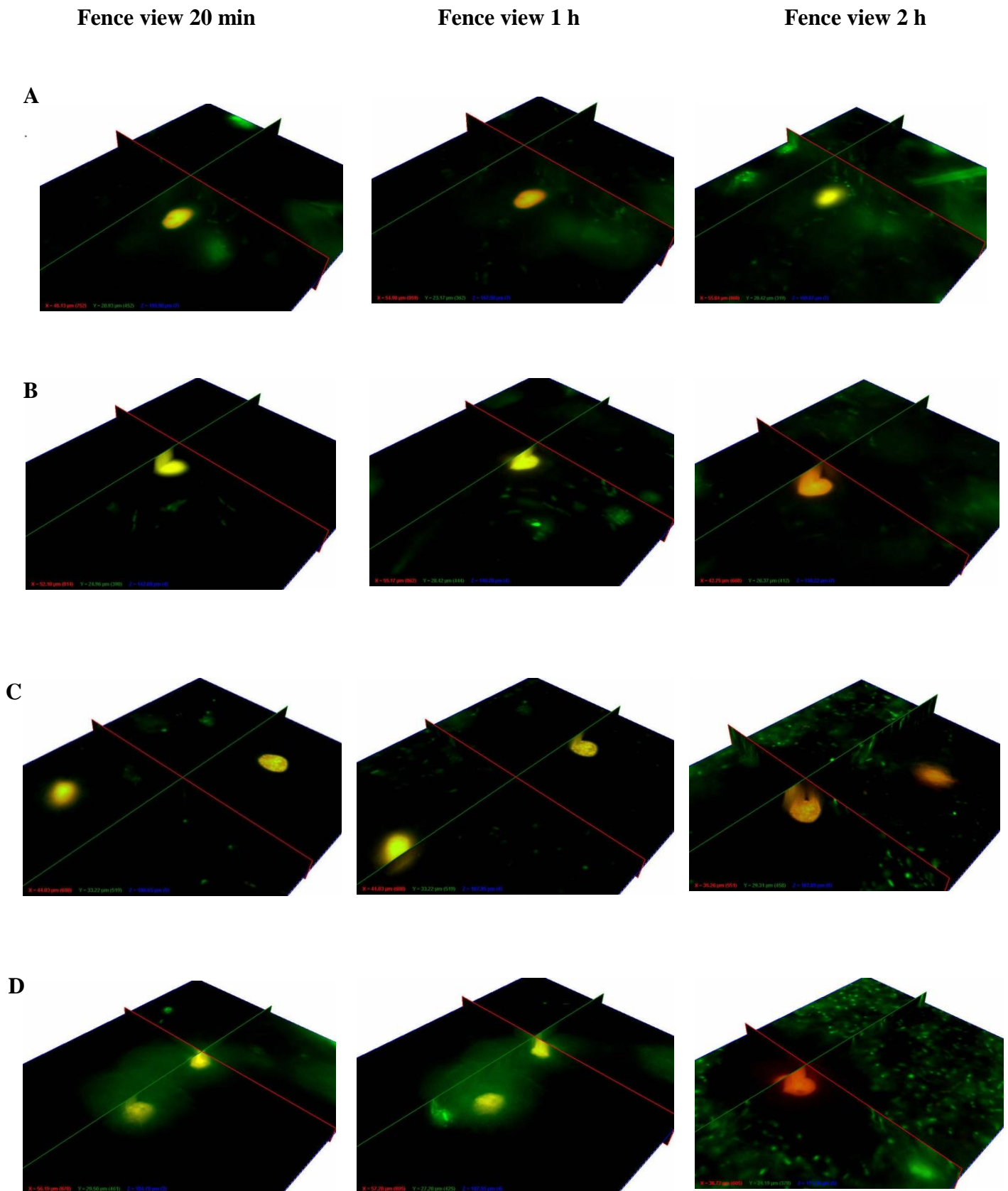


FIG. 5b. Fence view of adhesion of bacterial strains **A:** *L. equigenerosi* Le1, **B:** *L. reuteri* Lr1, **C:** *L. plantarum* 423 and **D:** *E. mundtii* ST4SA, pre-treated with pronase, to epithelium at 20 min, 1 h and 2 h.

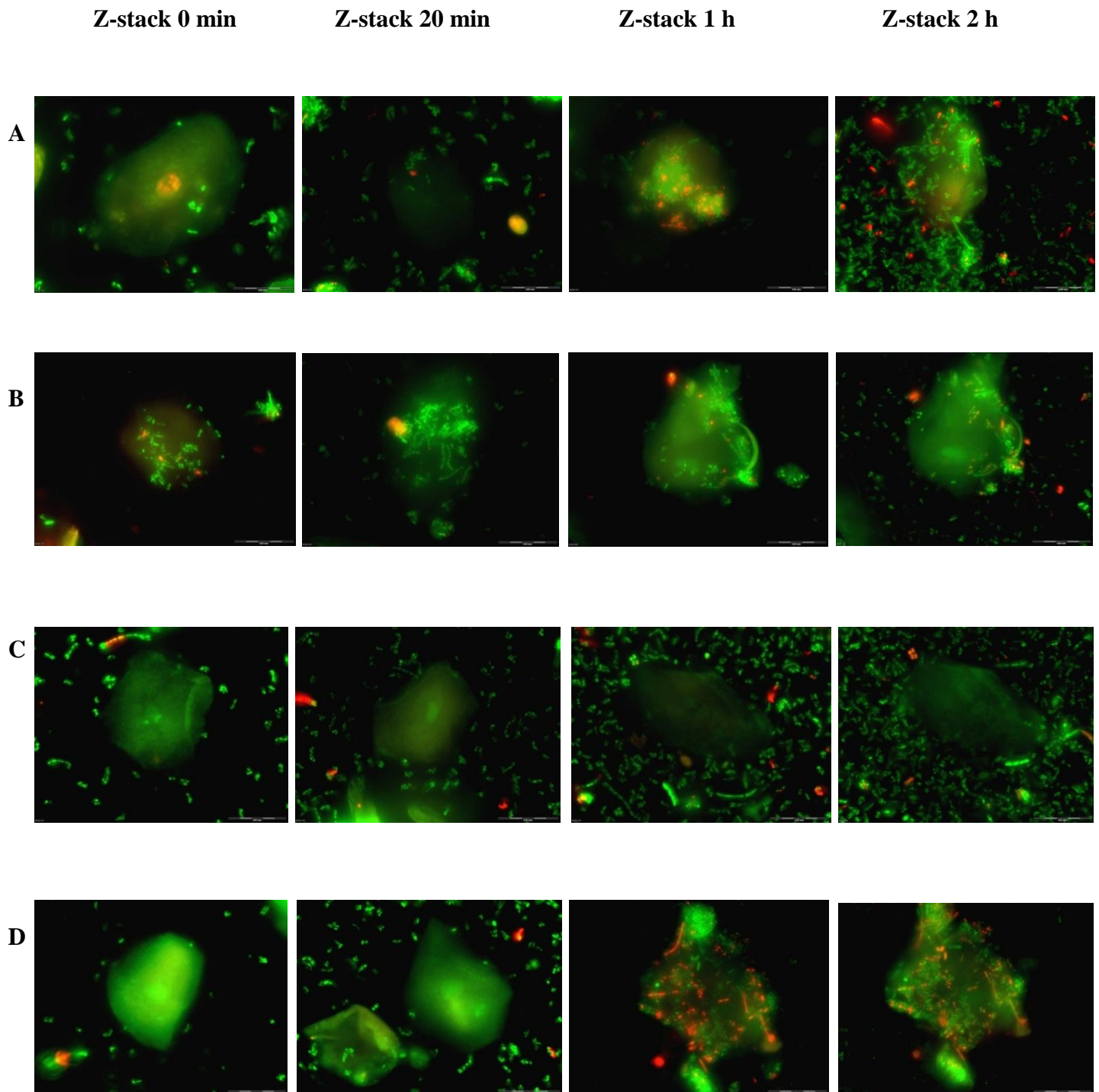


FIG. 6a. Adhesion of bacterial strains **A**: *L. equigenerosi* Le1 (27.5%), **B**: *L. reuteri* Lr1 (35.48%), **C**: *L. plantarum* 423 (20.33%) and **D**: *E. mundtii* ST4SA (100%), pre-treated with pepsin, to epithelium at 0 min, 20 min, 1 h and 2 h.

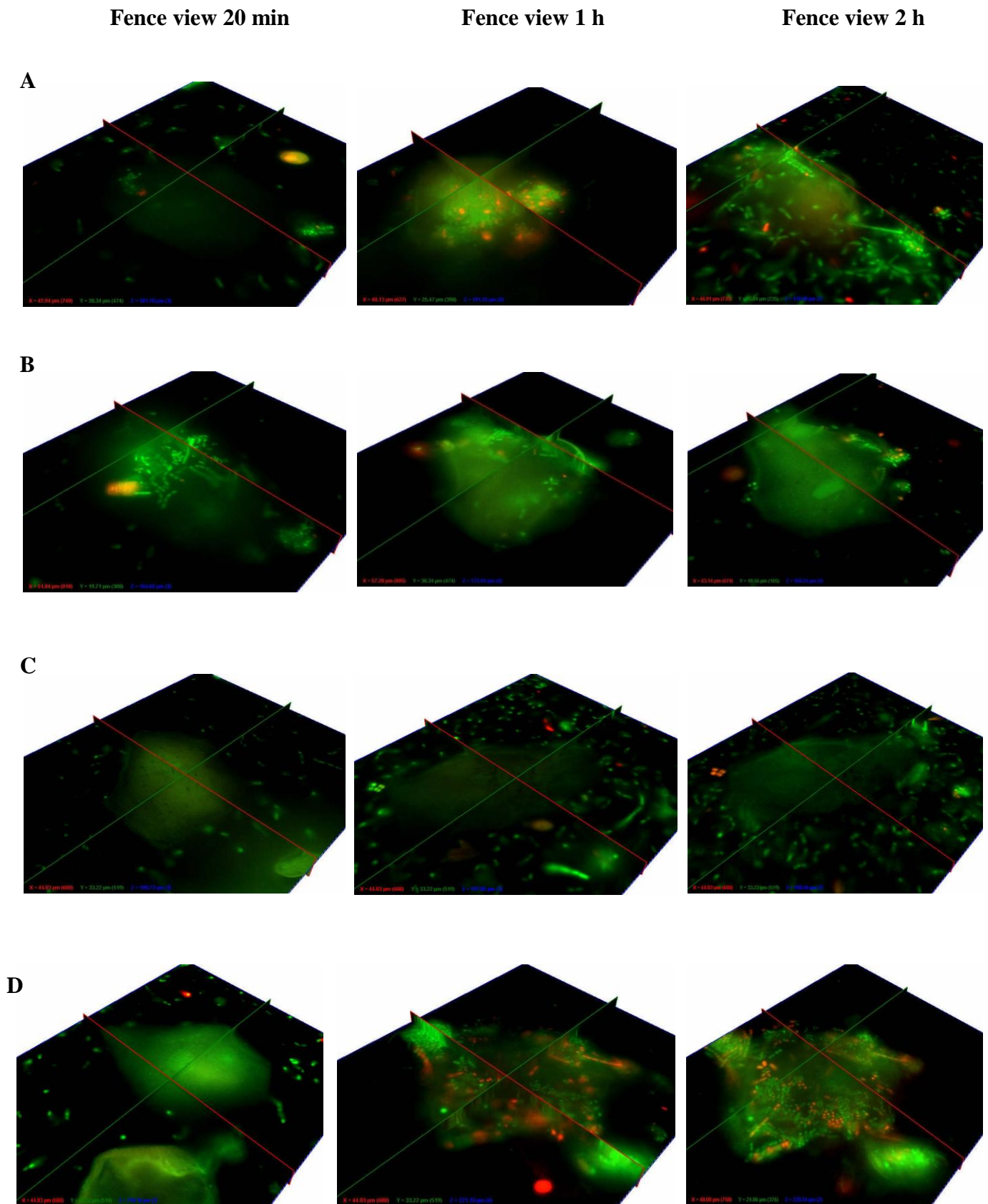


FIG. 6b. Fence view of adhesion of bacterial strains **A:** *L. equigenerosi* Le1, **B:** *L. reuteri* Lr1, **C:** *L. plantarum* 423 and **D:** *E. mundtii* ST4SA, pre-treated with pepsin, to epithelium at 20 min, 1 h and 2 h.

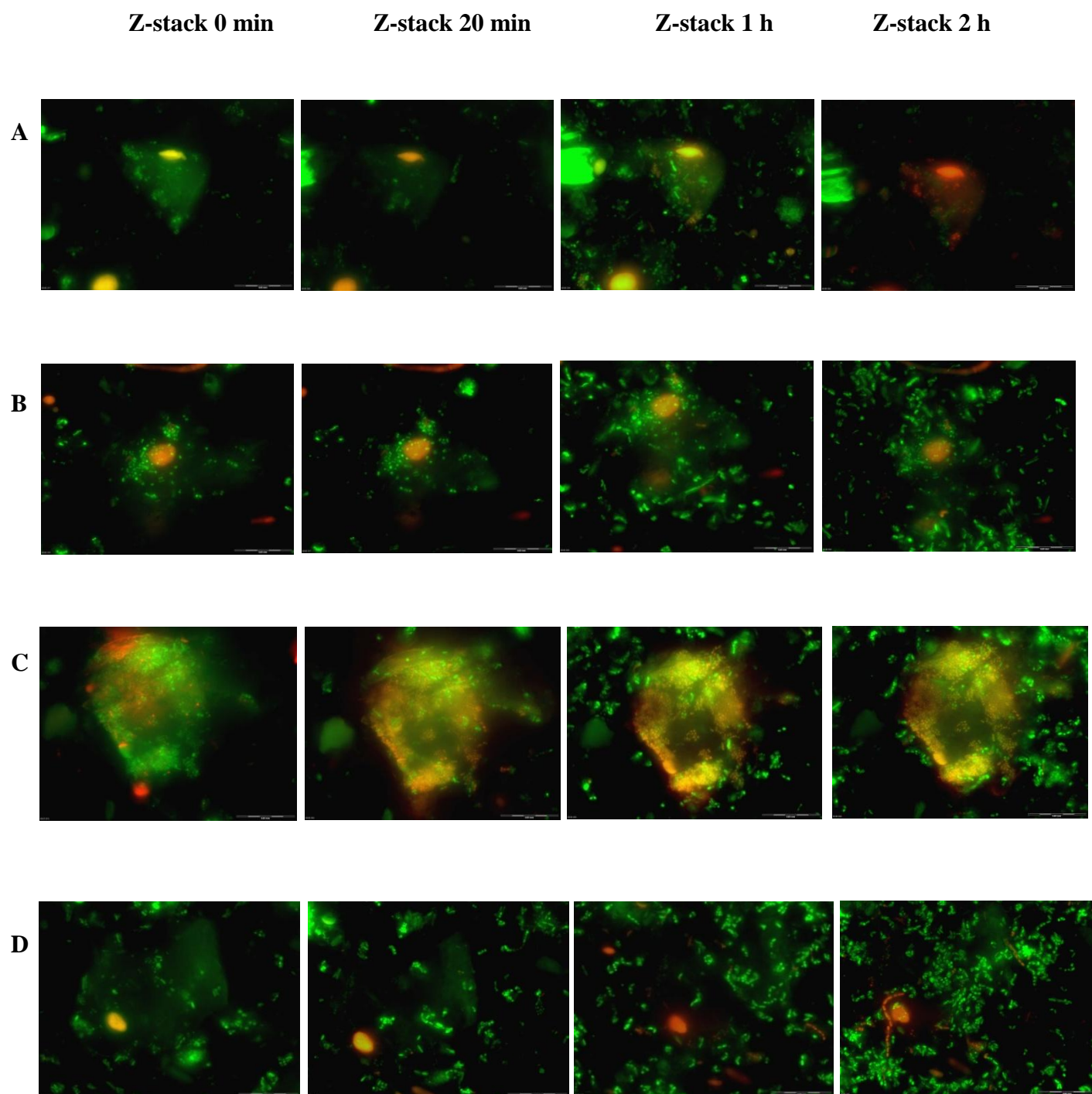


FIG. 7a. Adhesion of bacterial strains **A**: *L. equigenerosi* Le1 (MRS: 5.25% and DRCM: 3%), **B**: *L. reuteri* Lr1 (MRS: 1.6% and DRCM: 5.3%), **C**: *L. plantarum* 423 (MRS: 1.4% and DRCM: 7%) and **D**: *E. mundtii* ST4SA (MRS: 1% and DRCM: 1.7%) to epithelium in competition with *Clostridium* sp. C6 at 0 min, 20 min, 1 h and 2 h.

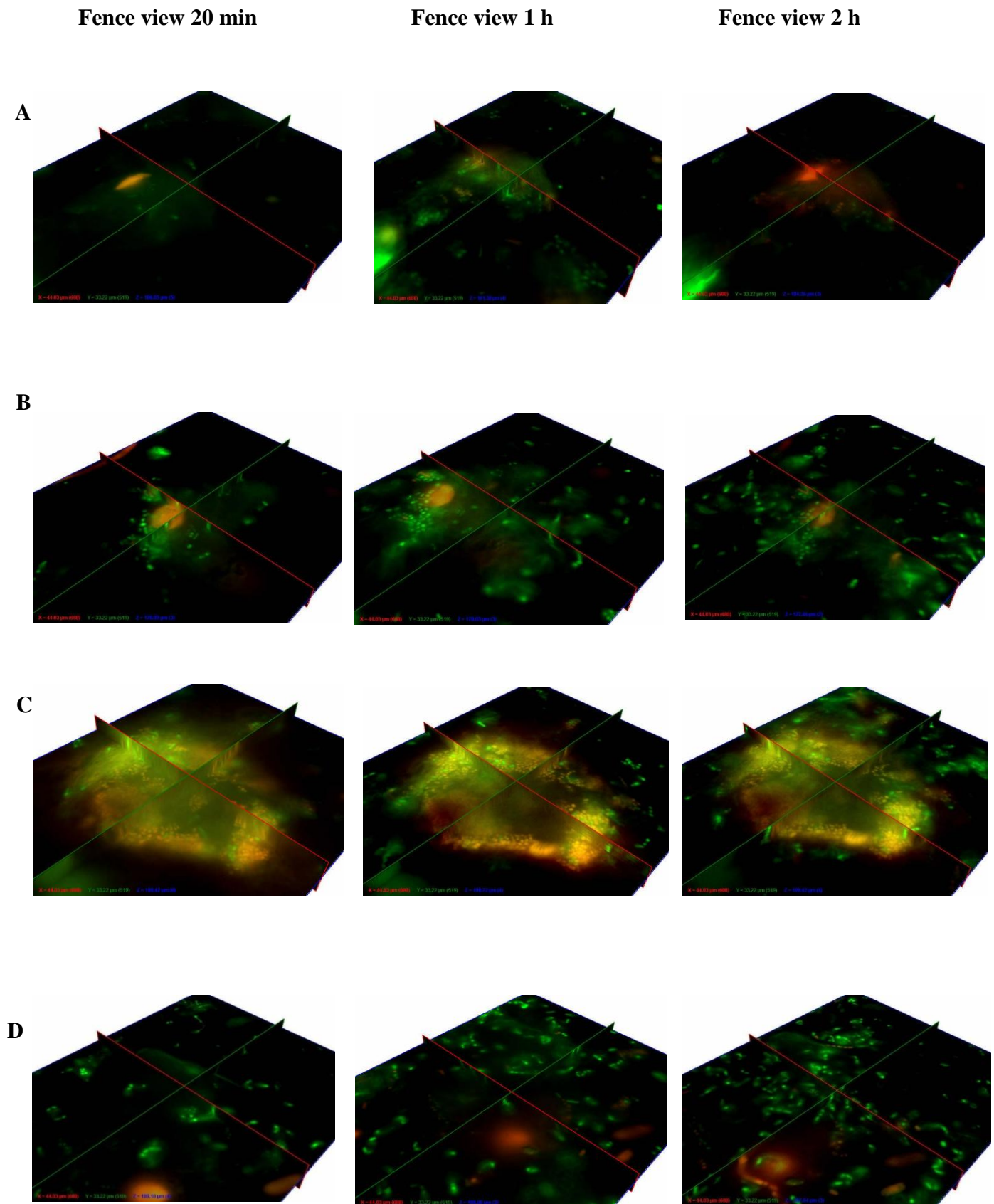


FIG. 7b. Fence view of adhesion of bacterial strains **A:** *L. equigenerosi* Le1, **B:** *L. reuteri* Lr1, **C:** *L. plantarum* 423 and **D:** *E. mundtii* ST4SA to epithelium in competition with *Clostridium* sp. C6 at 20 min, 1 h and 2h.

CHAPTER 4

Survival of lactic acid bacteria through the intestine and physiological changes recorded when administered to horses

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Survival of Lactic Acid Bacteria through the Intestine and Physiological Changes Recorded when Administered to Horses

MARLIE BOTHA¹, MARELIZE BOTES¹, CARINE SMITH² AND LEON M.T. DICKS¹

Department of Microbiology¹ and Department of Physiological Sciences², University of Stellenbosch, Stellenbosch 7600, South Africa.

An imbalance in microbiota in the equine digestive system causes gastro-intestinal disorders of which colic is the most common and responsible for most deaths. *Lactobacillus equigenersi* Le1 and *L. reuteri* Lr1 were isolated as the most predominant species from four experimental horses. Strains of these two species, together with bacteriocin-producing strains *L. plantarum* 423 and *Enterococcus mundtii* ST4SA, were administered orally to healthy horses for 10 consecutive days. The number of viable LAB, *Lactobacillus*, *Enterococcus*, *Clostridium* and *Salmonella* spp. in faecal samples was determined by plating onto selective growth media. Changes in the population of the genera were studied by using denaturing gradient gel electrophoresis (DGGE). Based on these results, *L. reuteri* Lr1 was contained the longest in the GIT, suggesting that it colonised the intestinal tract more efficiently compared to the other three species. *Clostridium* and *Salmonella* numbers were controlled by the four LAB strains administered. Total white blood cell counts remained stable during administration. Small, but tentative, increases in neutrophil and eosinophil numbers were recorded (lasting 3-4 days), suggesting that the LAB strains that were administered may elicit a mild, transient allergic reaction. Glucose, lactate and urea levels decreased during the administration period.

The equine gastro-intestinal tract has evolved to accommodate continuous grazing on a diet rich in fibre (58). The diet of stable horses, especially racehorses, contains high levels of starch to meet energy requirements (29, 46). Starch is not actively digested in the small intestine and most is past onto the hindgut to be fermented (29, 46) to lactate (3, 46) and volatile fatty acids (VFAs) (46). This lowers the pH in the hindgut (46) and favours the growth of lactic acid bacteria (LAB) that, in turn, ferments low molecular weight sugars which decreases the pH further (46). Lactobacilli and streptococci are predominant and tend to outgrow most other LAB in the GIT. At this low pH, Gram-negative bacteria are easily lysed and, if high numbers of pathogenic strains are present, high levels of endotoxins are released in the GIT (21, 38). These conditions are perfect for the onset of diseases such as colic, lactic acidosis (8, 21, 37, 49) and laminitis (22, 48). Antibiotics are generally used as growth promoters and to treat intestinal infections (26). The continued use of antibiotics may, however, distort the balance in natural microbiota and may lead to colic (4, 24, 25).

Commercially available probiotics are often administered to relief GIT disorders (45). By definition, probiotics are defined as a live microbial supplement that improves the microbial balance within the intestine (20). *Lactobacillus equi*, *Lactobacillus crispatus*, *Lactobacillus johnsonii*, *Lactobacillus reuteri* and *Lactobacillus salivarius* have been administered to horses to promote growth and treat diarrhoea (60). However, *Lactobacillus pentosus* WE7, isolated from horses, did not prevent diarrhoea when administered to neonatal foals (57). On the other hand, a combination of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus plantarum* and *Enterococcus faecium* controlled *Salmonella* infection in horses (56). *Enterococcus faecium* successfully colonized the GIT of rabbits and displayed probiotic properties (51). *Enterococcus faecium* effectively reduced the cell numbers of *Salmonella* and *Clostridium* spp. in horses (23). Not all LAB are safe, as *Lactobacillus delbrueckii*, *Lactobacillus fermentum*, *Lactobacillus mucosae*, *L. reuteri* and *L. salivarius* have been associated with equine laminitis (3, 43).

Lactobacilli have been isolated from the stomach, caecum, colon, rectum (46, 59) and hindgut (9, 13, 15, 31, 32). *Lactobacillus buchneri*, *Lactobacillus equigennerosi*, *Lactobacillus hayakitensis*, *Lactobacillus kitasatonis*, *L. delbrueckii*, *L. equi*, *L. johnsonii*, *L. mucosae*, *L. reuteri* and *L. salivarius* have been isolated from faeces (14, 40, 41, 42). The predominant species isolated from the faeces of thoroughbred horses are *L. equi*, *L. equigennerosi* and *L. hayakitensis* (41). *Enterococcus faecalis*, *Enterococcus mundtii* and *E. faecium* have also been isolated from faeces (31). *Clostridium* spp. have been isolated from the GIT (9, 28) and is considered part of the natural gut microbiota. High levels of faecal *Clostridium* have been associated with diseased horses (2, 5, 6, 33, 19, 23, 27, 34).

The use of PCR-denaturing gradient gel electrophoresis (PCR-DGGE) provides an overview of the bacterial diversity and the changes in a specific group of bacteria within, for example, a faecal sample (13, 15, 16, 55). PCR-DGGE was used to monitor the main changes in bacterial flora in the horse gastro-intestinal system, the presence of *Enterococcus durans*/ *E. faecium* was detected (39). In another study an abundance of enterococci strains were isolated from the horse rectum (11).

In this study, the main objectives were to develop a probiotic for horses that is able to survive passage through the GIT. In this study, the survival of *L. equigenerosi* Le1, *L. reuteri* Lr1, *L. plantarum* 423 and *E. mundtii* ST4SA in the GIT was monitored by culture- and molecular techniques. Changes in the microbial population were studied by comparing DNA banding patterns obtained from PCR-DGGE. Changes in blood aspartate aminotransferase, cholesterol, glucose, lactate and urea levels were also monitored.

MATERIALS AND METHODS

Animals. Four healthy crossbreed horses, between 3 and 16 years old (numbered A to D), stabled at the Welgevallen experimental farm of the University of Stellenbosch, were included in this study. Permission to perform the research was granted by the Ethical Committee (ethical clearance number 2009B03002). The horses were stabled separately in 9m² quarters and were fed high quality lucern, hay and a commercial energy-rich feed supplement twice a day. They were dewormed four weeks before the onset of the experiment.

Strains and culture conditions. The lactic acid bacteria selected for the study were two bacteriocin-producing strains, viz. *Enterococcus mundtii* ST4SA, isolated from soy beans (30) and *Lactobacillus plantarum* 423, isolated from traditionally fermented sorghum beer (54), and *Lactobacillus equigenerosi* Le1 and *Lactobacillus reuteri* Lr1, previously isolated from faeces of healthy horses (unpublished data). Strains ST4SA and 423 have previously been tested for probiotic properties (7, 47).

Ten ml cultures of each strain, grown in MRS broth (Biolab, Biolab Diagnostics, Midrand, South Africa), were each inoculated into 90 ml MRS Broth (Biolab) and incubated at 37°C for 18 h. The cultures were inoculated into separate 1 liter volumes of sterile molasses (10% w/v) and incubated for a further 18 h at 37°C. The number of viable cells in each culture was determined by plating onto MRS (Biolab) agar. All plates were incubated anaerobically at 37°C for 18 h.

Dosage. The lactic acid bacteria were administered by suspending the cells in 10% (w/v) molasses. Each suspension contained an equal number of each strain. The dosage was calculated based on 1×10^9 viable cells cfu per 50 kg bodyweight. Each horse received 20 ml of the cell suspension early morning and 10 h later another 20 ml. The experiment ranged over a period of 24 days, consisting of a before administration period (7 days), during administration (10 days), and after administration period (7 days).

Sampling and isolation of lactic acid bacteria. Faecal samples were collected from each of the horses seven days before administration of the lactic acid bacteria and on each successive day for the duration of the experiment. Serial dilutions of the samples were prepared in sterile saline, plated onto MRS agar (Biolab) and Lactobacillus Specific Agar (LBS) (Becton Dickinson, MD, USA), modified as described by Endo et al. (16). The plates were incubated anaerobically at 37°C for 48 to 72 h in flasks containing gas generating envelopes (Anaerobic system BR0038B, Oxoid Ltd., Basingstoke, Hants, UK). Enterococci were isolated by plating onto Enterococcus Specific Agar (Difco, Becton, Dickinson and Company, Le Pont de Claix, France), followed by incubation at 37°C for 48 h. Samples were also plated onto Differential Reinforced Clostridial Medium (DRCM) agar (Merck, Darmstadt, Germany) and incubated anaerobically at 37°C for 48-72 h. *Salmonella* spp. were selected for by plating onto Salmonella Shigella (SS) agar (Merck, Darmstadt, Germany), incubated aerobically at 37°C for 48 h.

Colonies were randomly selected from the MRS (Biolab) and LBS agar plates and inoculated into corresponding broth, supplemented with L-cysteine hydrochloride (500 mg/L) to sustain anaerobic conditions. Pure cultures were obtained by streaking onto MRS (Biolab) and LBS agar and then stored at -80°C in the presence of 40% (v/v) glycerol.

Identification of lactic acid bacteria. DNA was isolated from the bacteria using the ZR Fungal/Bacterial DNA kit (Zymo Research, CA, USA) and amplified by using the primers and conditions listed in Table 1. Reaction mixtures were prepared as used by Endo and Okada (12). The PCR amplification was as described by Walter et al. (55). Sequencing of the 16S rDNA was done using an automatic sequencer (ABI Genetic Analyzer 3130X1, Applied Biosystems, SA) and BigDye Terminator chemistry (Biosystems, Warrington, England). Sequences were subjected to Blast analysis on the GenBank.

PCR products were analyzed with DGGE (Denaturing Gradient Gel Electrophoresis) using a DCode System (Bio-Rad Laboratories, Hercules, CA, USA). The denaturing gradient of the 8% (w/v) polyacrylamide gels (acrylamide/bisacrylamide ratio, 37.5:1) ranged from 35% to 50%. In this case a 100% denaturant represents 40% formamide and 7 M urea. PCR products were prepared and

loaded onto the gels for the electrophoresis process that was performed at 70 V for 16 h in a Tris-acetate-EDTA buffer. Immediately after electrophoresis the gels were stained with SYBR Green I nucleic acid gel stain (BioWhittaker Molecular Applications, Rockland, ME, USA) for approximately 30 min. Clearly visible bands on the DGGE gels were excised with sterile toothpicks. DNA present within the excised gel pieces were amplified using the same primer sets and conditions used to amplify DNA for the DGGE procedure.

Blood analysis. Blood was drawn from the jugular vein of each of the horses on predetermined days before dosage with lactic acid bacteria and on specific days thereafter for the duration of the experiment. One blood sample was kept in EDTA, one sample was kept without an anticoagulant (SST tubes), and one sample stored in the presence of sodium fluoride. The SST tubes were centrifuged and the collected serum stored at -80°C. Samples with EDTA were used to determine full blood counts and white blood cell (WBC) counts, using the Celldyne 3700CS Haematology Analyser with Veterinary Software Package. Sodium fluoride tubes were used for glucose and lactate analysis. The aspartate aminotransferase (AST), glucose, cholesterol, lactate and urea levels were determined by Pathcare (4 Saffraan Avenue, Die Boord, Stellenbosch, Western Cape).

Statistical analysis. Statistical analysis of the blood biochemical profiles was done using two-way analysis of variance (ANOVA). Variability between groups was noted when $P \leq 0.05$ and the F-value was more than the F-crit value.

RESULTS

Cell numbers. Viable cell numbers are presented in Fig. 1. Total anaerobic LAB levels of horse A decreased from 10^8 to 10^7 CFU/g during administration, followed by an increase to 10^8 CFU/g after administration. *Clostridium* levels decreased from 10^7 to 10^6 CFU/g after administration, whereas *Salmonella* numbers decreased from 10^6 to 10^5 CFU/g during administration of the probiotic. The enterococci levels of horse B increased from 10^4 to 10^5 CFU/g after probiotic administration. *Salmonella* counts decreased from 10^5 to 10^4 CFU/g during administration but increased again to 10^5 CFU/g after administration.

Results of horse C showed that more lactobacilli (an increase from 10^5 to 10^6 CFU/g) were present within the faeces during and after administration. The lactobacilli levels of horse D during the preliminary decreased from 10^7 to 10^6 CFU/g after the administration period. Lactobacilli levels were higher (from 10^6 to 10^7 CFU/g) during the administration period, but lower (10^6 CFU/g) after

this period. *Salmonella* levels increased from 10^5 to 10^6 CFU/g during administration and decreased after administration to 10^5 CFU/g.

The overall outcome of the live faecal cell counts of all the horses is presented in Fig. 1e). Lactobacilli levels increased during administration from 10^5 to 10^6 CFU/g. Overall enterococci levels were higher after administration (an increase from 10^4 to 10^5 CFU/g). *Salmonella* and *Clostridium* numbers remained stable throughout the experiment at 10^5 and 10^6 CFU/g, respectively.

Identification of bacteria. The most predominant faecal lactobacilli strains identified from the experimental horses was *L. equigenerosi* Le1, presenting a 100% homology with its closest relative (GenBank accession no. AB425935) and *L. reuteri* Lr1, with 98% homology to a reference strain in GenBank (accession no. CP002844).

DGGE profiles. Faecal lactobacilli DGGE profiles for horses A-D are presented in Fig. 2. Faecal samples from horse A always contained *L. equigenerosi* Le1. A band resembling *L. plantarum* 423 was not detected on the DGGE, therefore the strain might have not been present in the faeces. The lowest CFU level that can be detected by the DGGE is 10^3 CFU/g faeces (13). The only day that *L. reuteri* Lr1 was present in the faeces was on day 9.

Horse B had the same result as horse A. *L. reuteri* Lr1 was once again only detected on day 9 of the experiment and *L. equigenerosi* Le1 was always present.

The DGGE profile of horse C during the experiment again corresponded with that of horses A and B. *L. reuteri* Lr1 being excreted on day 9 and *L. equigenerosi* Le1 was always prominent within the faeces. On day 16 it seemed that *L. plantarum* 423 might have been detected on the gel photo, therefore might have been present within the faeces.

Faeces from horse D on days 11 and 14 of the study definitely contained *L. plantarum* 423. Once again *L. reuteri* Lr1 was present on day 9 and *L. equigenerosi* Le1 was always present within the faeces.

E. mundtii ST4SA could not be detected on the faecal enterococci DGGE profiles of the horses during the preliminary and secondary experiment (data not shown). The reason could be that the enterococci levels within the faeces were too low to be detected.

Blood analysis. Statistical analysis of blood biochemical profiles during the experimental period can be seen in Table 2. In Fig. 3, the WBC counts are shown with no statistical differences between the days before, during and after administration. During the experiment the red blood cells (RBC),

haemoglobin and haematocrit showed variability. Overall aspartate aminotransferase (Fig. 4a), cholesterol (Fig. 4b), glucose (Fig. 4c), lactate (Fig. 4d) and urea (Fig. 4e) levels for the second experiment are presented in the figures. Statistical variation was seen between samples of blood cholesterol, glucose, lactate and urea.

DISCUSSION

Overall the total faecal anaerobic LAB levels during the experiment remained at 10^7 CFU/g (Fig. 1e), with no significant changes in their levels before, during and after the administration of the probiotic. Interestingly, horse A was the only horse that had 10^8 CFU/g before and after the administration of the probiotic (Fig. 1a). During the administration period the levels dropped to 10^7 CFU/g. Total anaerobic bacterial count in foal faeces before administration of a host-specific *Lactobacillus* probiotic was 10^9 /g and after was 10^{10} /g (60). An increase in total faecal anaerobic bacteria has been speculated to be linked to an imbalanced gastro-intestinal (GI) microbial environment in animals suffering from a disease (23). Administered probiotic LAB that were unable to colonize within the gut will however also be excreted and might have an effect on faecal numbers. The overall stable levels of these bacteria within the faeces therefore indicate a healthy GI microbial environment throughout the study.

Seventy five percent of the probiotic LAB used in this study were lactobacilli and therefore it was important to note the differences in faecal lactobacilli during the experimental period. The overall outcome was a constant level of 10^6 CFU/g during the experimental period (Fig. 1e). Lactobacilli numbers in faeces previously studied by Endo et al. (13) ranged between 10^7 and 10^9 CFU/g, whereas another study by Endo et al. (15) on South African horses indicated that 10^6 to 10^8 CFU/g lactobacilli were present. According to Fey and Sasse (18), the normal amount of faecal lactobacilli should be at least 10^5 CFU/g. Faecal lactobacilli levels fell within previously described levels and therefore no irregularities were observed during the study.

Enterococci levels overall before and during administration were 10^4 CFU/g but increased to 10^5 CFU/g after administration (Fig. 1e). Administered enterococci might have been retained for a while before being excreted. Horse A (Fig. 1a) had the highest level of faecal enterococci (10^5 CFU/g), the rest of the horses had 10^4 CFU/g throughout the experimental period, except horse B (Fig. 1b) that had an increase after administration to 10^5 CFU/g. Enterococci levels in faeces recorded by Lauková et al. (31) were 10^1 - 10^5 CFU/g.

Overall, *Clostridium* levels were stable at 10^6 CFU/g. Three of the horses had this number of live clostridia within the faeces but horse A had higher levels of 10^7 CFU/g (data not shown). Since horse A's background indicates a regular struggle with GI disorders, the stable *Clostridium* levels were a good report on the administered probiotic. Faecal *Clostridium* numbers were stable and at times decreased during the experimental period. *Lactobacillus plantarum* PCA 236 administered to dairy goats had a similar effect on faecal *Clostridium* levels as these levels decreased by day 7 of administration from 10^7 to 10^6 CFU/g (35).

Salmonella levels in horses A and B decreased from 10^6 to 10^5 and from 10^5 to 10^4 CFU/g, respectively, during administration of the probiotic (data not shown). Horse C had consistent faecal levels (10^4 CFU/g) during the whole experiment, whereas horse D had an increase (10^5 to 10^6 CFU/g) in numbers during administration, followed by a decrease in numbers (back to 10^5 CFU/g) after administration (data not shown). Overall, a slight decrease in *Salmonella* levels was observed during administration of the probiotic and remained at these levels after administration. As these levels were to some degree under control by the probiotic, the result of this experiment on *Salmonella* was effective.

L. equigenerosi Le1 and *L. reuteri* Lr1 were the predominant isolates from faeces of the experimental horses used in this study. The DGGE analysis of the faecal microbial populations revealed that *L. equigenerosi* was the predominant lactobacilli since this *Lactobacillus* sp. was always present within the faeces before, during and after administration of the probiotic. This result corresponds with research done by Morita et al. (41). Therefore no definite conclusions can be made about retention of *L. equigenerosi* Le1 by the horses when this native LAB was administered, even though faecal lactobacilli levels of horses A and D increased during administration.

It is assumed that *L. reuteri* Lr1 was retained by the horses up to day 9 of the preliminary study, because of the fact that this *Lactobacillus* sp. only appeared within faeces on this day (Fig. 2). Interestingly, on day 9 horse A had an increase in lactobacilli from 10^7 to 10^8 CFU/g and horses C and D had an increase from 10^6 to 10^7 CFU/g (data not shown). Horse B already had an increase in lactobacilli (from 10^6 to 10^7 CFU/g) on day 8 and remained at 10^7 CFU/g for day 9 (data not shown).

L. plantarum 423 was detected on days 14 and 16 of the experiment in the faeces of horse D but could not be detected on the DGGE of any of the other horses. This species might have been present but at very low levels. On the days of detection within the faeces there were no increases in faecal lactobacilli levels of these horses (data not shown), suggesting that low levels of *L. plantarum* 423 were excreted.

Very low levels of enterococci within the faeces of the experimental horses during the experiment made this microbial population very hard to study with DGGE analysis. DGGE analysis performed did not pick up the presence of *E. mundtii* ST4SA within the faeces during the experimental period (data not shown). A reason might be that this *Enterococcus* sp. was retained till after the administration period as levels increased from 10^4 CFU/g (before and during the administration) to 10^5 CFU/g after administration. There is no way of knowing as these levels are very low and difficult to detect with DGGE analysis.

From the overall DGGE analysis outcomes, it is speculated that all four LAB strains could have been retained by the horses for a brief period when administered. It is, however, certain that *L. reuteri* Lr1, after being administered, was retained for at least 8 days before being excreted as this result was seen in all four horses during the same experiment.

The general health of the horses during both experiments was excellent but to obtain information on any blood abnormalities, indicating a negative reaction towards the probiotic, blood analysis was performed. On day 7 there was a very small increase in neutrophil levels. Neutrophils are phagocytic WBC that play an important part in the innate immunity of the host (10) as these cells cause acute inflammation (50) when they come into contact with invading organisms. The corresponding reaction of a change in neutrophil levels would be a change in lymphocyte levels (50). No changes in lymphocyte levels were seen but on day 11 there was a slight increase in these counts. However, no statistical difference in terms of neutrophil or lymphocyte levels was detected during the experimental period.

Eosinophil levels were lower on day 7 and higher on day 11, but these differences were not statistically significant. Eosinophils are involved in allergic responses towards foreign material such as parasites (36). Basophil levels were elevated on day 11 but with no statistical significance. This occurred 1 day after administration of the probiotic and, therefore, the probiotic cannot be isolated as the reason for this response.

During the experiment there were statistical variances in RBC counts, haemoglobin and haematocrit levels (Table 2). All of these parameters had the same trend namely, levels rising slightly from day 0 till day 3 from where these levels gradually decreased again (data not shown). In contrast, these parameters increased when an enterocin-A producing strain of *Enterococcus faecium* EK13 was administered to piglets (52).

Glucose levels were mildly elevated on day 3 but immediately started decreasing after this day (Fig. 4c). On day 3, lactate levels were also higher but like the glucose levels, started decreasing after

this day (Fig. 4d). Higher lactate levels combined with increased RBC, haemoglobin and haematocrit levels has been seen in horses when exercising (61). Blood glucose levels in horses were higher at the beginning of a training period than the rest of the training period (53). High glucose levels can cause colic (19). Decreasing glucose levels during the administration of the probiotic in this study, therefore, is a positive effect.

High RBC, haemoglobin, haematocrit, glucose and lactate levels initially seen during this experiment might, therefore, be linked to the experimental horses undergoing exercise during this time. Nervous horses will have elevated heart rates and this is also a factor to be taken into consideration. High blood lactate levels can often lead to lactic acidosis (1). During the experimental period lactate levels decreased gradually, thus the LAB, when administered, did not cause an increase in blood lactate levels and therefore is a good attribute.

Aspartate aminotransferase levels (Fig. 4a) in this study did not change and this corresponds to results from Strompová et al. (52). In a horse affected with acute colic, the aspartate aminotransferase levels were double the levels from our study (44). Even the resting aspartate aminotransferase levels of horses in a study by Tateo et al. (53) was very high compared to our results but in this case the age of the horses played a role.

Cholesterol levels started decreasing slightly by day 11 of this study (Fig. 4b), data obtained by the Strompová et al. (52) study had the same tendency except that cholesterol levels started decreasing by day 7. Urea levels decreased gradually till day 11 from where it increased slightly on day 16 (Fig. 4e).

CONCLUSIONS

The combination of lactic acid bacteria administered to horses in this study controlled the number of *Clostridium* and *Salmonella*. Lactobacilli, in particular, *L. reuteri* Lr1 was retained after administration for a brief period. Enterococci were also retained till after the administration period. *L. equigennerosi* Le1 was constantly present within the faeces confirming the predominance of this species within horses. There was no negative effect on the general health of the horses. During administration of the probiotic, glucose, lactate and urea levels decreased. High levels of these parameters are dangerous as the host can develop fatal GI disease for example colic (19). This probiotic for administration to horses has potential and seems promising based on the positive results obtained from the *in vivo* study. Future research should include more horses within the

study and establish the fact that these probiotic lactic acid bacteria colonize within the gut of the host.

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TABLE 1. Primers used during this study

Primers	Sequence (5' to 3')	Annealing Temperature (°C)	Reference
16S rDNA			
8F	CACGGATCCAGACTTTGATYMTGGCTCAG	54	(17)
1512R	GTGAAGCTTACGGYTAGCTTGTTACGACTT		
DGGE			
Lac1	AGCAGTAGGGAATCTTCCA	61	(55)
Lac2GC	CGCCCGGGGCGCGCCCCGGGCGGCCCGGG GGCACCGGGGGATTTCACCGCTACACATG		(55)
Lac3	AGCAGTAGGGAATCTTCGG	61	(12)

TABLE 2. Statistical analysis of blood biochemical profiles during the experimental period
(Adapted from 61)

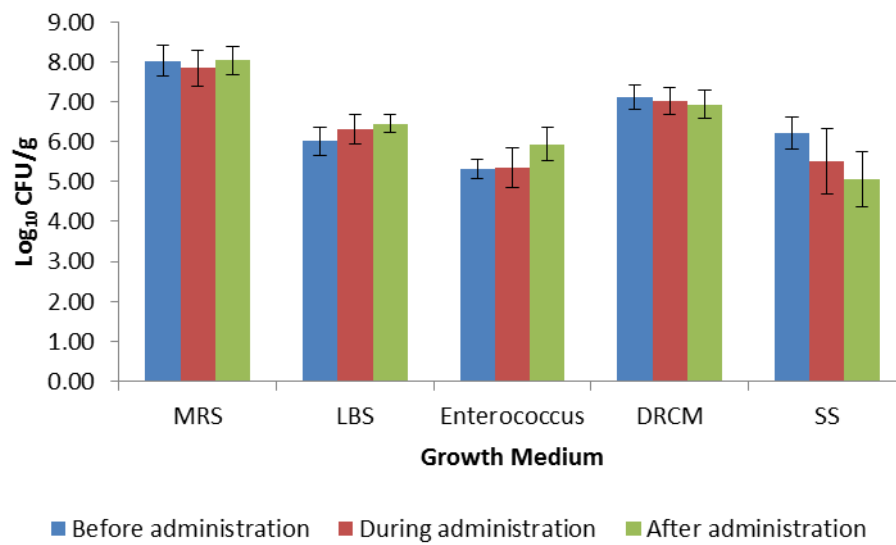
Variables with units	Mean and SD			Variance (<i>P</i> values)		
	$\bar{X}_B \pm SD_B$	$\bar{X}_D \pm SD_D$	$\bar{X}_A \pm SD_A$	B vs. D	B vs. A	D vs. A
WBC ($10^9/L$)	7.65 ± 1.80	7.50 ± 0.89	7.26 ± 0.90	*	*	*
NEU ($10^9/L$)	3.29 ± 0.77	3.56 ± 0.18	3.42 ± 0.01	*	*	*
LYM ($10^9/L$)	3.34 ± 0.87	3.13 ± 0.10	2.76 ± 0.37	*	*	*
MONO ($10^9/L$)	0.37 ± 0.09	0.36 ± 0.00	0.34 ± 0.00	*	*	*
EOS ($10^9/L$)	0.13 ± 0.07	0.11 ± 0.04	0.11 ± 0.07	*	*	*
BASO ($10^9/L$)	0.12 ± 0.11	0.12 ± 0.01	0.22 ± 0.09	*	*	*
RBC ($10^{12}/L$)	7.71 ± 0.65	7.74 ± 0.39	7.15 ± 0.33	*	**	*
HGB (g/dL)	13.5 ± 0.6	13.5 ± 0.71	12.4 ± 0.74	*	**	**
HCT (%)	64.9 ± 2.9	64.9 ± 2.53	60.5 ± 2.47	*	**	*
PLT ($10^9/L$)	204 ± 56	200 ± 12.90	183 ± 35.53	*	*	*
AST (UI/L)	291 ± 31	291 ± 1.06	283 ± 10.43	*	*	*
Cholesterol (mmol/L)	2.33 ± 0.15	2.34 ± 0.05	2.24 ± 0.16	*	**	**
Glucose (mmol/L)	5.40 ± 0.79	5.50 ± 0.39	4.65 ± 0	*	**	**
Lactate (mmol/L)	0.83 ± 0.33	0.94 ± 0.16	0.72 ± 0.15	*	*	**
Urea (mmol/L)	5.25 ± 3.28	4.65 ± 0.02	4.28 ± 0.32	**	*	*

SD: Standard deviation, B: Before administration, D: During administration, A: After administration. WBC: White blood cells, NEU: Neutrophils, LYM: Lymphocytes, MONO: Monocytes, EOS: Eosinophils, BASO: Basophils, RBC: Red blood cells, HGB: Haemoglobin, HCT: Haematocrit, PLT: Platelets, AST: Aspartate aminotransferase.

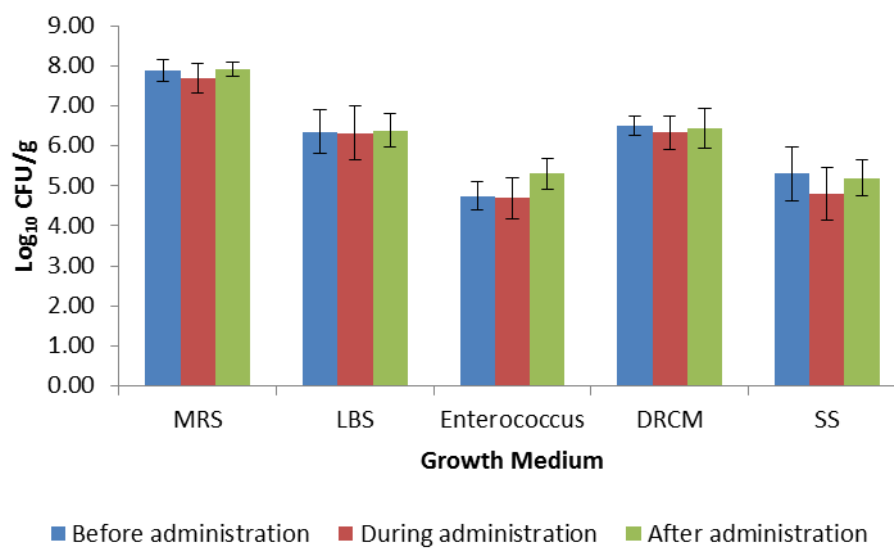
* $P > 0.05$

** $P \leq 0.05$

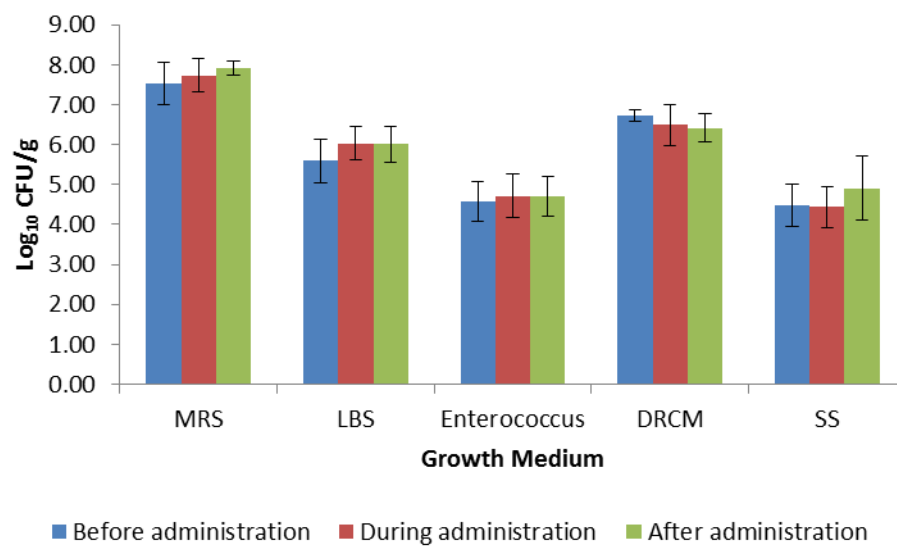
a)



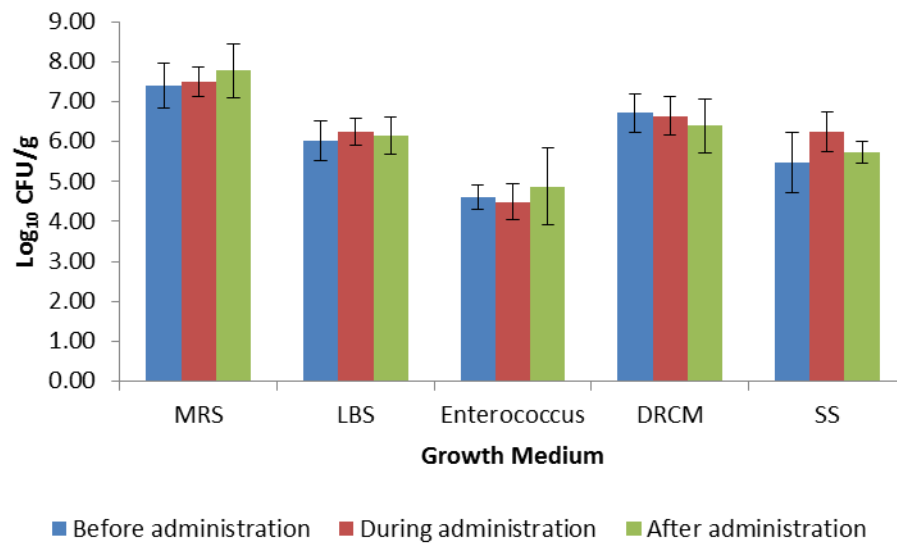
b)



c)



d)



e)

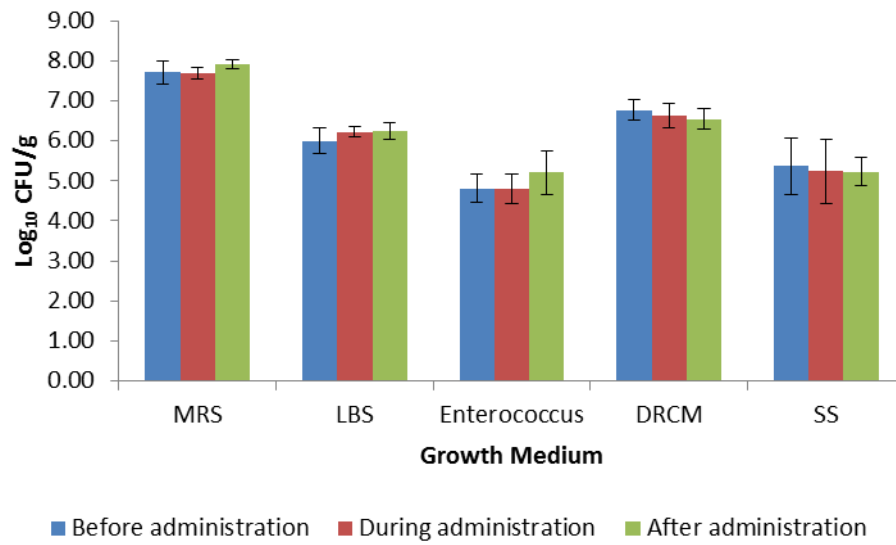


FIG. 1. Live faecal cell counts of a) Horse A, b) Horse B, c) Horse C, d) Horse D and e) the overall cell counts.

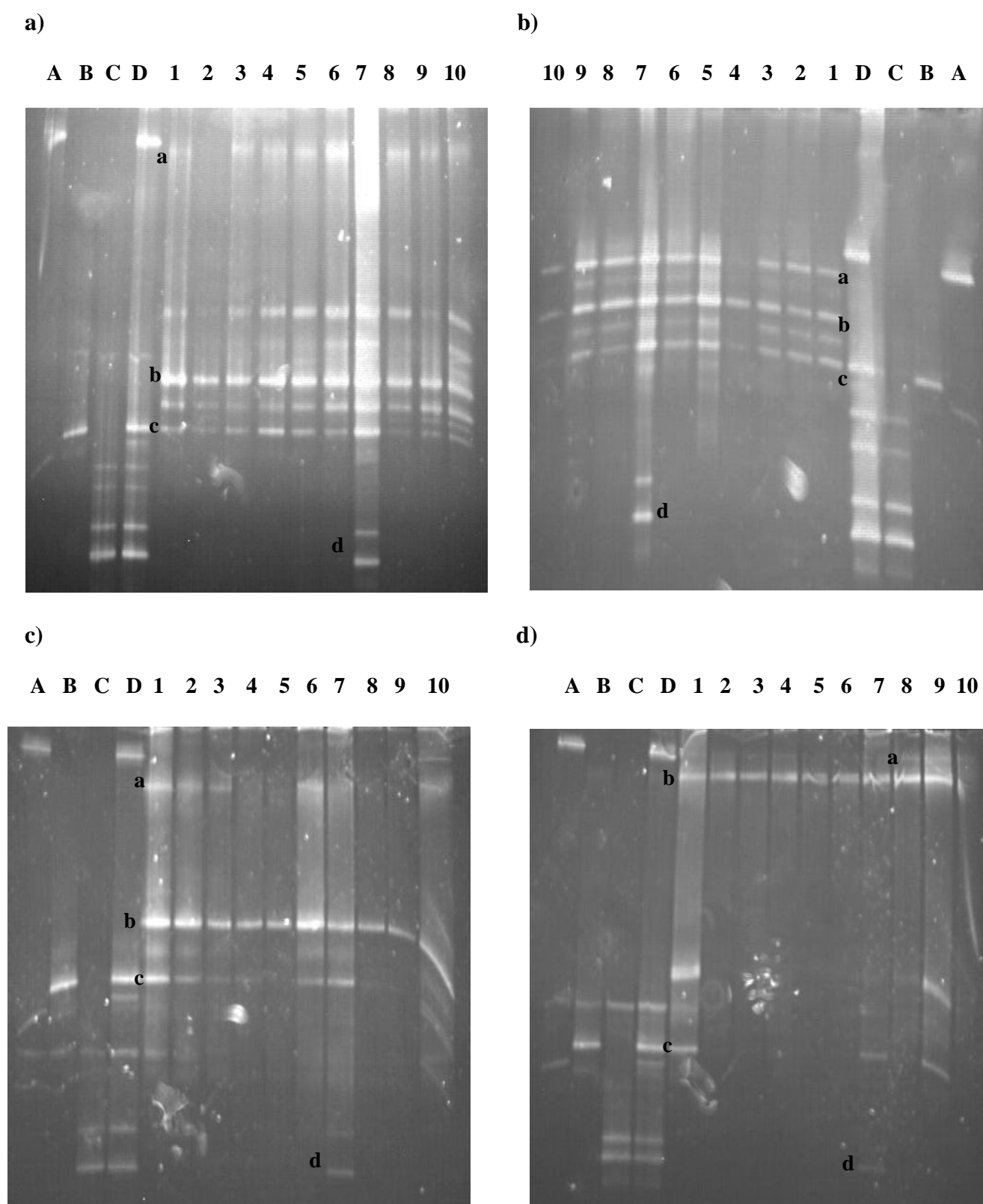
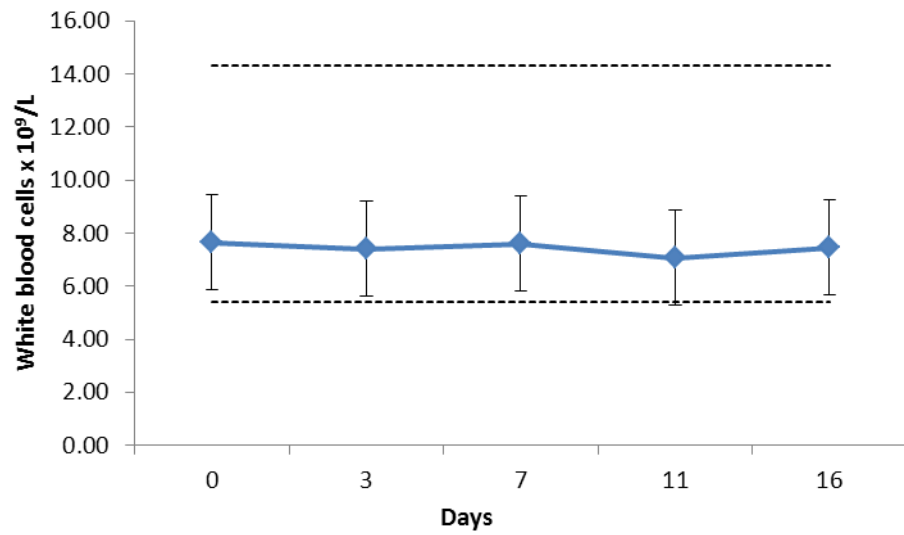
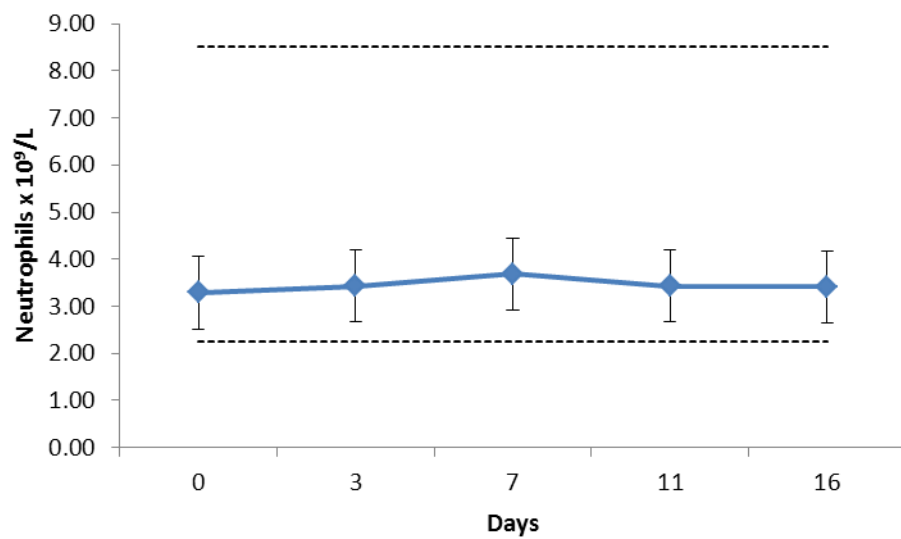


FIG. 2. Faecal lactobacilli DGGE profiles. a) Horse A, b) Horse B, c) Horse C and d) Horse D. **A:** *L. plantarum* 423, **B:** *L. equigenerosi* Le1, **C:** *L. reuteri* Lr1 and **D:** Ladder of A-C. Lanes **1-3:** Before administration (Day -6, -4 and 0), **4-7:** During administration (Day 2, 4, 7 and 9) and **8-10:** After administration (Day 11, 14 and 16). For a) to c): Bands **a-c** are *L. equigenerosi* and band **d** is *L. reuteri*, and for d) band **a** is *L. plantarum*, **b** and **c** are *L. equigenerosi* and **d** is *L. reuteri*.

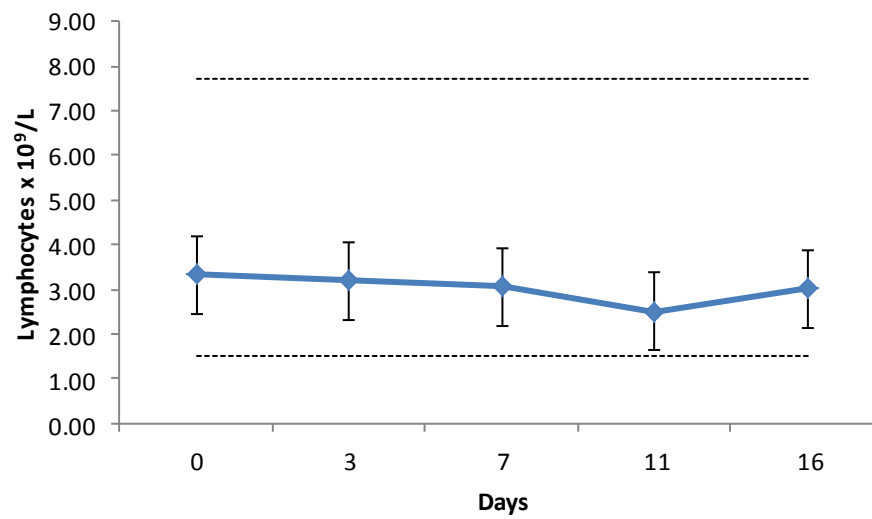
a)



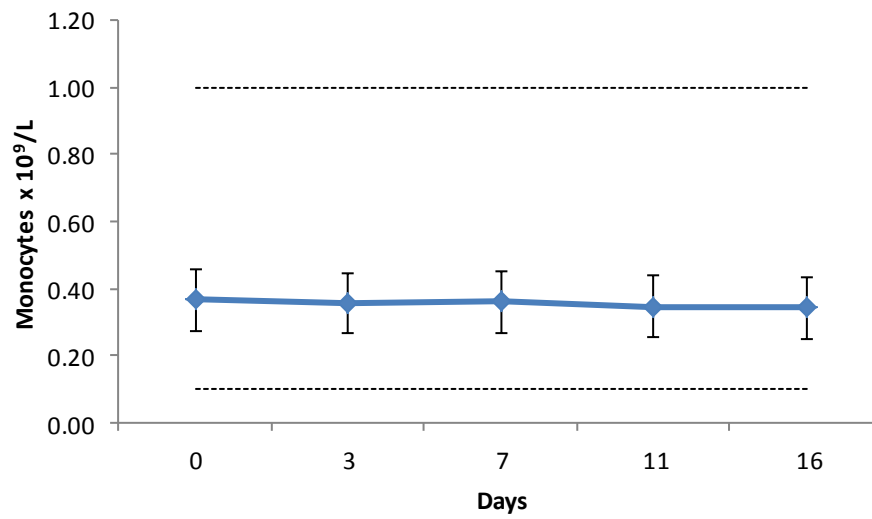
b)



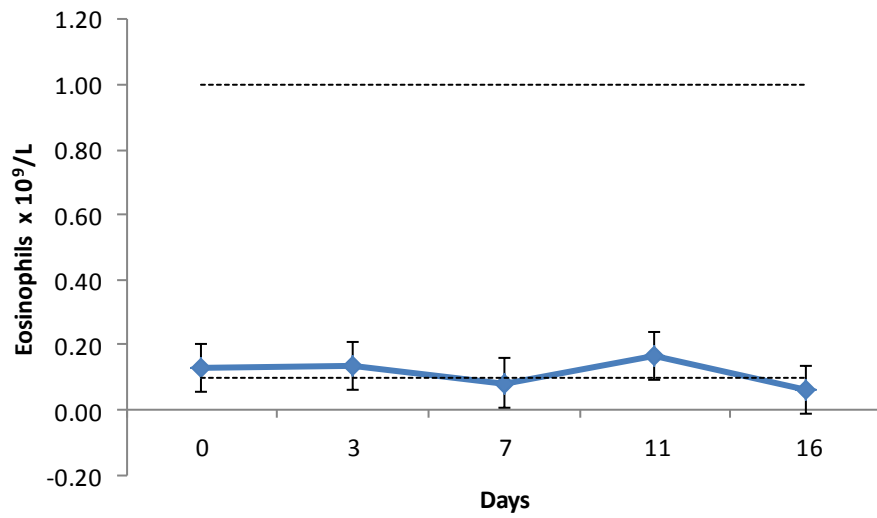
c)



d)



e)



f)

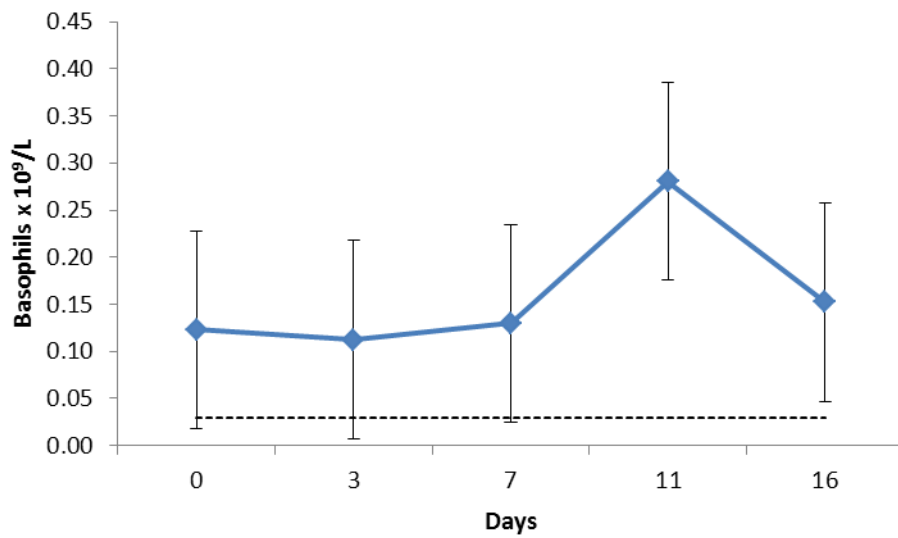
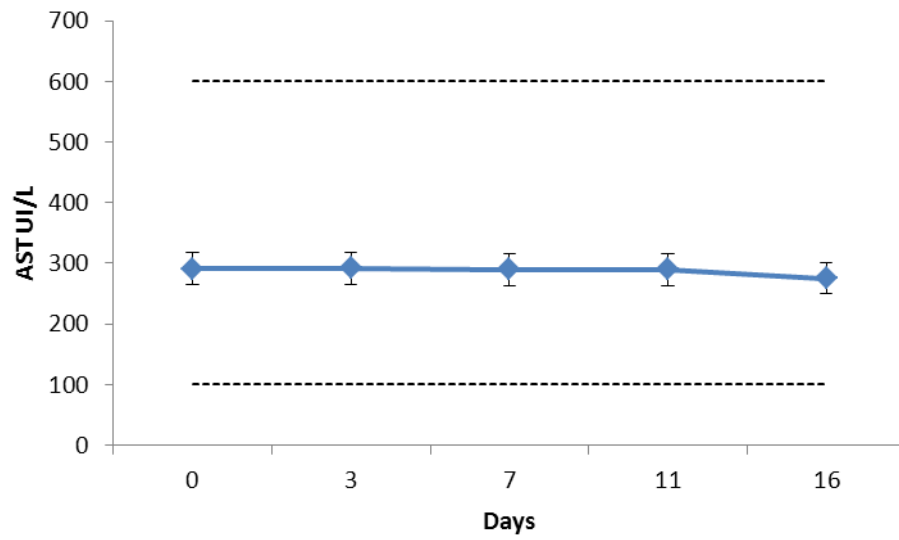
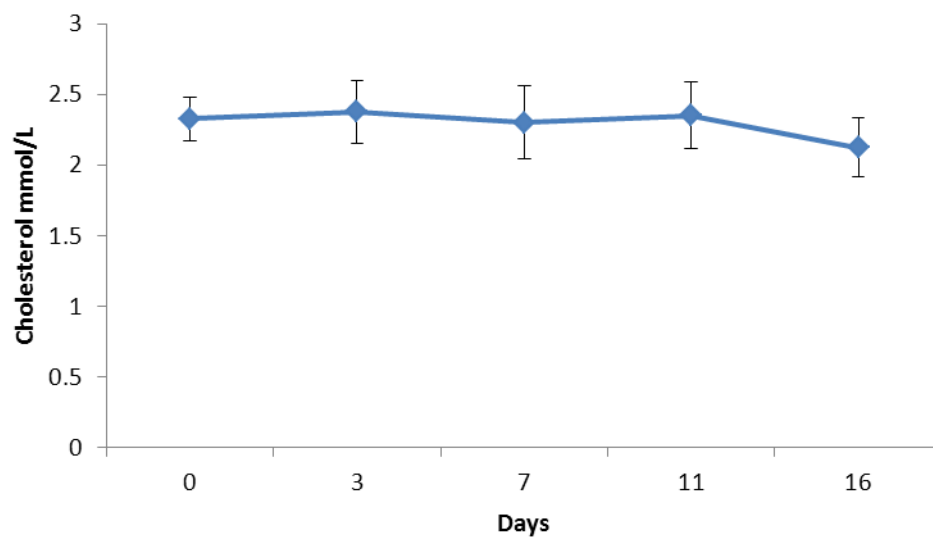
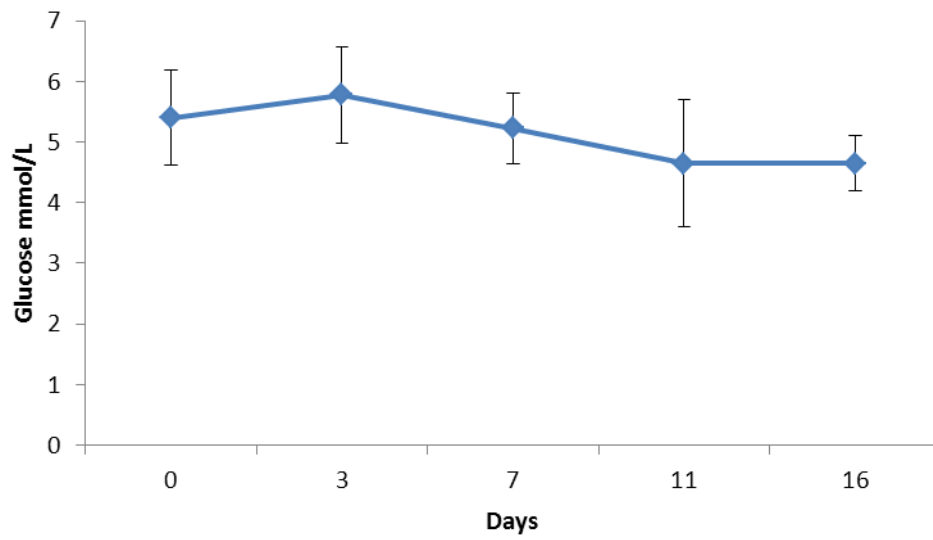


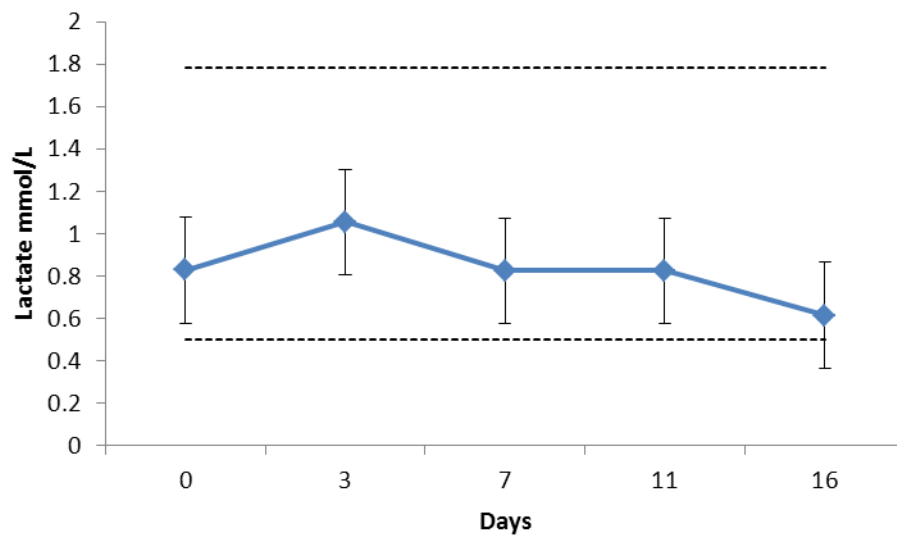
FIG. 3. a) White blood cells, b) neutrophils, c) lymphocytes, d) monocytes, e) eosinophils and f) basophils counted during the experimental period. Baseline values are indicated with the dashed line (61).

a)**b)**

c)



d)



e)

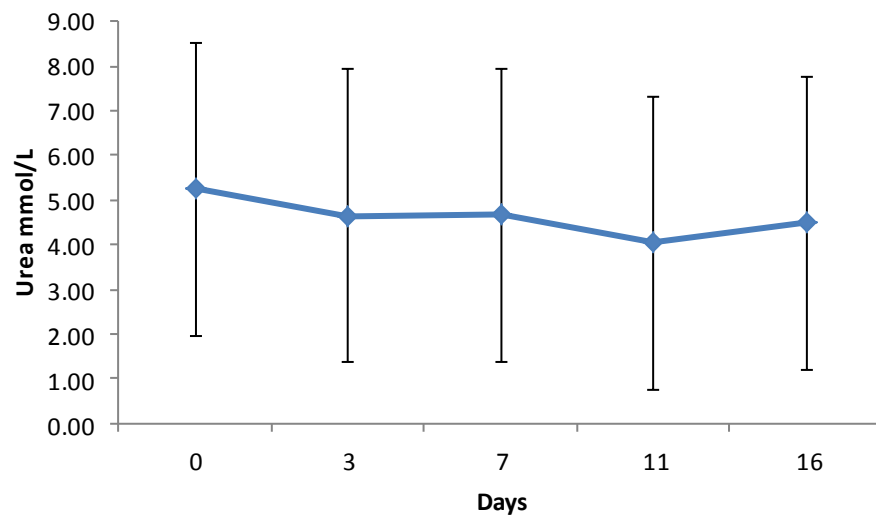


FIG. 4. a) Aspartate aminotransferase, b) cholesterol, c) glucose, d) lactate and e) urea levels measured during the experimental period. Baseline values are indicated with the dashed line (61).

CHAPTER 5

General Discussion and Conclusions

GENERAL DISCUSSION AND CONCLUSIONS

Little research has been done on the natural microbial communities of the equine GIT. An imbalanced microbial community often leads to the onset of colic, which is the most common and, very often, fatal disease (2, 9, 12). Probiotics are beneficial in terms of maintaining a balanced microbial community within the GIT of the host (7). LAB have to meet certain criteria to be administered as probiotics, of which the safety aspects of the strain(s) are the most important (8). Strains need to survive gastric pH, withstand bile salts, colonize (adhere to) epithelium or mucus and compete with pathogens for adhesion (8).

The main focus of this study was to develop a probiotic for horses to maintain a well-balanced microbial community within the GIT and, therefore, improve the general health of the host. Two predominant lactobacilli (*Lactobacillus equigenersi* Le1 and *Lactobacillus reuteri* Lr1) were isolated from horse faeces. The two strains were used in combination with the human probiotic strains *L. plantarum* 423 (isolated from sorghum beer) and *Enterococcus mundtii* ST4SA (isolated from soy beans). All four strains survived low pH and bile salts that simulated conditions of the GIT. *L. reuteri* Lr1 was especially resistant to very low pH (pH 2). Strains were able to tolerate low pH to a degree (from pH 3) when pH levels were lowered with the addition of lactic acid. As other LAB within the GIT produce lactic acid, these strains will be able to survive these conditions.

All four of the selected strains were screened for the presence of virulence genes (adhesion to collagen (Ace), aggregation substance (AS), cytolysin (Cyl), non-cytolysin (β hemolysin III) and vancomycin A,B and C) as this is an important factor to be evaluated as these strains might be potentially pathogenic (13). None of the strains, with the exception of *E. mundtii* ST4SA, had any of the virulence genes that were screened for. *E. mundtii* ST4SA have three of these virulence genes but these genes might not be expressed (1). Adhesion to epithelium or mucus allows the bacteria to modulate an immune response (14) or occupy adhesion sites that might have been adhered to by pathogenic bacteria (11). Cell surface hydrophobicity, auto-aggregation and co-aggregation of each strain were calculated to predict whether or not cells would adhere to epithelium, and whether or not these would be able to co-aggregate with a horse pathogen (*Clostridium* sp. C6). In practice, these values are a prediction and do not necessarily apply directly. *L. reuteri* Lr1 and *E. mundtii* ST4SA for example had 0% hydrophobicity but still adhered to viable epithelium cells. *L. equigenersi* Le1 and *E. mundtii* ST4SA invaded viable epithelium cells. *E. mundtii* ST4SA lost the ability to invade the cells after being treated with pronase. This treatment, together with pepsin treatment of the strains did not affect adhesion to epithelium.

Co-aggregation with pathogens are also important, LAB are allowed to produce antimicrobial peptides like bacteriocins in close proximity and inhibit pathogen growth (15). The two native strains (*L. equigenerosi* Le1 and *L. reuteri* Lr1) had the best co-aggregation potential with *Clostridium* sp. C6. The best competitive exclusion capability was also observed with *L. equigenerosi* Le1.

In vivo studies are the next step in the evaluation of a probiotic. To establish whether or not administered strains were retained, live faecal counts on selective media were determined. From faecal counts it seems that lactobacilli and enterococci administered were retained for a while by the hosts. Lower and stable levels of *Clostridium* spp. and *Salmonella* spp. were observed during administration of the probiotic. *Clostridium* spp. and *Salmonella* spp. levels within faeces are of particular importance as these bacteria can be potentially pathogenic (6, 10).

DGGE analysis of faecal samples was necessary to determine the shift in microbial populations (3, 4, 5, 16). From the analysis, it was clear that *L. reuteri* Lr1 in particular was retained by the horses for at least 8 days of administration before being excreted.

Based on full blood counts and additional analysis, there was no negative response towards the probiotic by any of the experimental horses. There were no statistically significant changes in the white blood cell counts during administration. During the administration period, glucose, lactate and urea levels decreased. Very high levels of these parameters are dangerous to the host as fatal GI disease for example colic may develop (6).

There is a lack of probiotics with success in horses on the market. This combination of strains is therefore a good potential probiotic as *in vitro* and *in vivo* evidence proves. More research on these strains might still be required.

In vitro evaluation of the selected strains suggests that *L. equigenerosi* Le1, *L. reuteri* Lr1, *L. plantarum* 423 and *E. mundtii* ST4SA in combination have potential to be administered as an equine probiotic. These strains are able to survive horse GIT conditions and these strains do not present to be potentially virulent. Adhesion to epithelium cells were relatively good (the best adhesion was 8%) and the two native strains have good co-aggregation potential with *Clostridium*, that is a horse pathogen. Competitive exclusion of this *Clostridium* was successfully done by all of the strains but *L. equigenerosi* Le1 was the most promising.

Horses looked healthy (skin condition was good, weight was normal, etc.) and no sign of disease was observed throughout the administration period, ranging over a period of two months. Horses retained *L. reuteri* Lr1 for 8 days of administration. No blood abnormalities were observed.

In future research, there has to be adhesion experiments performed on lower GIT epithelium cells of horses. Information on the reason as to why these strains invade the cells is necessary to obtain information on the mechanisms and action of LAB with it comes to epithelium cells and therefore the intestine. A bigger *in vivo* study is recommended, with more horses as these hosts are unique and differ considerably in every aspect.

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